# The Pleckstrin and Sec7 domain-containing gene as a novel epigenetic modification marker in human gastric cancer and its clinical significance

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Received August 15, 2014; Accepted October 1, 2014

DOI: 10.3892/ijo.2014.2736

**Abstract.** The Pleckstrin and Sec7 domain-containing (PSD) gene has been recently found to participate in the progression of several types of cancer. In the present study, we identified PSD as a candidate tumor suppressor gene silenced through epigenetic modification in gastric cancer (GC). PSD mRNA expression and DNA methylation were evaluated by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) and methylation-specific PCR in GC cell lines and tissue samples. Involvement of histone modification in GC cell lines was examined by chromatin immunoprecipitation assay. We also used an siRNA-mediated approach to knock down the PSD gene in SGC7901 cells, which was utilized to detect the role of PSD in GC progression, followed by analysis of cell apoptosis and invasion. PSD gene expression was reduced in all GC cell lines compared with GES1 (an immortalized normal gastric cell line). In addition, PSD expression was markedly downregulated in gastric carcinoma tissues when compared to adjacent non-tumor tissues. Our data also indicated that PSD mRNA and protein levels were associated with tumor differentiation and lymph node metastasis. Aberrant DNA methylation status and histone modification were also found in GC cell lines. Enhanced gene expression was detected when the HGC27, AGS and BGC823 GC cell lines were treated with the DNA-demethylating agent 5-aza-2'-deoxycytidine. However, treatment with trichostatin A, a histone deacetylase inhibitor, had no effect on PSD expression in any of the GC cell lines. Suppression of PSD by siRNA led to enhanced SGC7901 cell invasion. The depletion of PSD expression inhibited cell proliferation and decreased apoptosis in SGC7901 cell lines. Knockdown of the PSD expression decreased caspase-3 and -7 protein levels in SGC7901 cells. PSD gene may function as a tumor suppressor in GC suggesting a vital role for DNA methylation and histone modification in PSD silencing. PSD expression might be a useful biomarker for epigenetic-based GC early diagnosis and may lead to the identification of new targets for pharmacological intervention.

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*Key words:* PSD, gastric carcinoma, histone modification, DNA methylation

## Introduction

Gastric cancer (GC) is a significant health problem worldwide, and is the second most common cause of cancer-associated mortality (1). It is well known that GC results from combination of a complex series of genetic and epigenetic events. It is reported that epigenetic alteration contributes to the progression of GC via regulating the expression of tumor suppressor genes (TSGs). The silencing of TSGs by these epigenetic regulators is recognized as a vital mechanism in GC formation (2-4). Therefore, a better understanding of the abnormal epigenetic modification of GC is key for determining how to inhibit GC progression effectively.

DNA methylation and histone modifications are key players in epigenetic modification, and appear to be linked to each other (5,6). Early studies have shown that DNA methyltransferases (DNMTs), together with the methyl-CpG-binding protein MECP2, localize to DNA-methylated promoters and recruit a protein complex that contains histone deacetylases (HDACs) and histone methyltransferases (7-9). Extinction of DNA methylation affecting H3 methylation and other histone modifications have also been found in *Arabidopsis* and human cells (10,11). These studies suggest DNA methylation induces chromatin structural changes through alteration of histone modifications. DNA methylation and histone modifications likely have a mutually reinforcing relationship and both are required for stable and long-term epigenetic silencing of TSGs. Aberrant DNA methylation and histone modifications lead to downregulation or silencing of TSGs and are often found in CpG-rich sites, known as CpG islands, located in the promoter region of many TSGs (12-14). The 5'-end of the PSD gene contains five CpG islands, suggesting that its expression may be controlled by epigenetic modification.

In our previous studies (15,16), genome-wide analysis of histone modifications, using a chromatin immunoprecipita-

tion microarray (ChIP-chip) investigated inactive genes in GC, with the aim of identifying targets of genes controlled by histone modifications. We identified the PSD gene as a possible TSG in GC. PSD is a guanine nucleotide exchange factor for ADP-ribosylation factor 6 (15), which regulates the membrane trafficking of small G proteins (16). It is located on human chromosome 10q24 and encodes a 71-kDa protein (17). Okada *et al* (18) reported that DNA methylation at the PSD promoter is more frequently methylated in ulcerative colitis (UC)-associated colorectal cancer tissues compared to non-neoplastic UC epithelia. In addition, PSD mRNA is positively correlated with the methylation status of PSD, however, little is known about the association between epigenetic alteration and PSD expression in GC.

In the present study, we investigated PSD expression in GC cells and tissues, and aimed to verify whether decreased PSD expression in GC is related to DNA methylation and repressive histone modification at the PSD promoter. Four GC cell lines and one normal gastric cell line were used to examine PSD mRNA expression and epigenetic alteration. In addition, 40 GC tissue specimens and 40 corresponding non-malignant gastric tissues were used to observe the mRNA and protein expression of PSD, and its clinical significance. Suppression of PSD in SGC7901 cells by siRNA transfection was utilized to detect the function of PSD in GC progression.

## Materials and methods

*Cell culture and treatment with epigenetic agents*. GES1, an immortalized normal gastric cell line, was obtained from the Oncology Institute of China Medical University (Shenyang, China). Four human GC cell lines, HGC27, AGS, BGC823 and SGC7901 were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). These cells were cultured in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and incubated at 37˚C in a humidified 5% $CO_2$ atmosphere. Four GC lines were incubated in culture medium with 5 $\mu$M DNMT inhibitor 5-aza-2'-deoxycytidine (DAC; Sigma, St. Louis, MO, USA) for 3 days, and 0.3 $\mu$M of the histone deacetylase inhibitor trichostatin A (TSA; Sigma) for 1 day. The time, dose and sequence of DAC and/or TSA were based on previous studies (19,20).

*Tissue samples*. Human GC samples were collected from 40 patients who underwent gastrectomy at Cancer Institute of China Medical University (Shenyang, China) between January 2009 and June 2011. All GC cases were pathologically confirmed. Non-malignant gastric tissues that were ≥5 cm away from the tumor were obtained from the patients. This study was approved by the Institutional Review Board of China Medical University.

*Chromatin immunoprecipitation (ChIP) assay*. Five million cells were crosslinked with 1% formaldehyde for 10 min at 37˚C, and then 0.125 M glycine was added to stop the crosslinking. After washing with ice-cold PBS, the cell pellets were resuspended in lysis buffer, and sonicated to generate 200-1,000-bp DNA fragments. The lysate was then divided into three fractions. The first lysate was precipitated using antibodies against Lys-9 trimethylated histone H3 (05-1242; Millipore, Billerica, MA, USA), Lys-9 acetylated histone H3 (07-352; Millipore) or Lys-4 trimethylated histone H3 (07-472; Millipore) at 4˚C overnight. The second lysate was incubated with normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a negative control. The third lysate was used as an input control. We added protein G-Sepharose beads to collect the immunoprecipitated complexes and left them to incubate for 1 h at 4˚C. After washing, the beads were treated with RNase (50 mg/ml) for 30 min at 37˚C and then proteinase K overnight. The crosslinks were then reversed by heating the sample at 65˚C for 6 h. DNA was extracted by the phenol/chloroform method, ethanol precipitated, and resuspended in 20 $\mu$l water.

*PCR analysis of immunoprecipitated DNA*. A total of 2 $\mu$l of immunoprecipitated DNA, DNA input control and negative control were used for PCR. The following primer set for PCR were designed to amplify the overlapping fragments of 190 bp along the PSD promoter: sense: 5'-gactggctt ctgtcgtcctc-3' and antisense: 5'-ggcagacagtaagagcctgg-3'. PCR products were subjected to 2.5% agarose gel electrophoresis at 120 V for 40 min and quantified using the Fluor Chen 2.0 system (Bio-Rad, Hercules, CA, USA). For quantitation, PCR amplification was performed on an ABI 7700 real-time PCR (Applied Biosystems, Foster City, CA, USA). PCR conditions included an initial denaturation step of 4 min at 95˚C, followed by 35 cycles of 5 sec at 95˚C, 30 sec at 60˚C and 20 sec at 72˚C. Quantitative ChIP-PCR values were normalized against values from a standard curve constructed using input DNA that was extracted for the ChIP experiment. The ChIP real-time PCR experiments were repeated three times.

*RNA extraction and real-time reverse transcriptase polymerase chain reaction (RT-PCR)*. Total RNA was extracted from cells and tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The quality and concentration of RNA were measured by ultraviolet absorbance at 260 and 280 nm ($A_{260}/A_{280}$ ratio) and checked by agarose gel electrophoresis individually. Total RNA was reverse transcribed into cDNA using an Expand Reverse Transcriptase kit (Takara, Dalian, China). Expression of PSD mRNA was detected using real-time PCR with the following program: 95˚C for 30 sec and 35 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The reaction mixture contained 12.5 $\mu$l SYBR Green (Takara), 1 $\mu$l each primer, 2 $\mu$l cDNA, and 8.5 $\mu$l diethylpyrocarbonate (DEPC)-treated water. Primers used were 5'-CTGGGCAAGAACAATGACTTC-3' (sense) and 5'-GAGGACAGGGCTTCAGGATT-3' (antisense) for PSD; and 5'-CATGAGAAGTATGACAACAGCCT-3' (sense) and 5'-AGTCCTTCCACGATACCAAAGT-3' (antisense) for glyceraldehyde-3-phosphate dehydrogenase (GADPH). The negative control used DEPC-treated water to replace cDNA templates for every PCR. The PSD level was expressed as Ct after normalization to the levels of GAPDH mRNA. The experiment was done in triplicate.

*PSD gene knockdown by siRNA*. Three pairs of the PSD siRNA sequence were designed and synthesized by GenePharma (Shanghai, China). As a result of relative effectiveness and

stability, the following siRNA sequence was selected in our experiments: 5'-CCAAGCUCAGGGUGUUUTT-3', 5'-AAA CACCCUGAGAGCUUGGTT-3'; it was transfected into SGC7901 cells. A non-silencing siRNA sequence was used as a negative control (5'-UUCUCCGAACGUGUCACGUTT-3', 5'-ACGUGACACGUUCGGAGAATT-3'). siRNA transfections were performed according to the manufacturer's instructions. SGC7901 cells were seeded in 6-well plates in medium containing 10% serum 24 h before the experiment. PSD or negative control siRNA of 100 pmol was diluted in 250 $\mu$l Opti-MEM I medium. Diluted siRNA was mixed with diluted Lipofectamine 2000 for 20 min. The oligomer-Lipofectamine complexes were applied to the SGC7901 cells. Following transfection, the PSD mRNA levels were assessed 48 h later.

*Methylation-specific PCR (MSP).* Genomic DNA was extracted from cells and tissues with phenol-chloroform-isoamyl alcohol and collected by ethanol precipitation. The bisulfite treatment was performed using the EZ DNA Methylation-Gold kit (Zymo Research, Los Angeles, CA, USA) according to the manufacturer's protocol. The CpG map of the PSD promoter and the location of primers used in this study were analyzed by http://www.urogene.org/methprimer/index1.html (21). We found five CpG islands in the PSD promoter region. The primers for the methylated PSD CpG island were 5'-GTTG TAGGGAAGCGGTTC-3' (sense) and 5'-CGACCACGAAA AAAAACC-3' (antisense). The primers for the unmethylated PSD CpG islands were 5'-AGGGTTGTAGGGAAGTGG TTT-3' (sense) and 5'-CAACCACAAAAAAAAACCTA-3' (antisense). Peripheral blood cell DNA from healthy adults treated with SssI methyltransferase (New England Biolabs, Ipswich, MA, USA) and untreated DNA were used as positive and negative controls, respectively. PCR products were separated by electrophoresis on 2% agarose gels.

*Western blotting.* Total protein was extracted from cells and tissues in a lysate buffer: 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Nonidet P40, 0.5% sodium deoxycholate and phenylmethylsulfonyl fluoride (all from Beyotime Institute of Biotechnology, Shanghai, China). Each sample (60 $\mu$g) was electrophoresed in 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking with 5% BSA in Tris-buffered saline-Tween-20 (20 mM Tris-HCl, 500 mM NaCl, 0.05% Tween-20) for 2 h at room temperature and then incubated with primary antibodies for PSD (1:500 dilution; Novus International, Littleton, MA, USA), caspase-3 (1:500 dilution; Millipore), caspase-7 (1:500 dilution; Millipore) or β-actin (1:2,000 dilution; ZSGB-Bio, Beijing, China) overnight at 4˚C. The next day, after incubation with a 1:2,000 dilution of secondary antibodies at 37˚C for 2 h, and after washing, the immunoreactive protein bands were visualized using an electrochemiluminescence (ECL) detection kit (Thermol Biotech, Rockford, IL, USA). The ratio between the optical density of the protein of interest and β-actin was calculated as the relative content of the protein detected. Each experiment was repeated three times.

*Wound healing assay.* Cells were plated in 6-well plates and maintained in RPMI-1640 medium containing 10% fetal calf serum. A wound was created in the center of the cell monolayer by a sterile plastic pipette tip. The cells were allowed to migrate for 24 h. Images were taken at 0, 12 and 24 h after wounding to assess the ability of the cells to migrate into the wound area using an inverted microscope (IX-71; Olympus, Tokyo, Japan).

*Matrigel invasion assay.* Approximately $5x10^4$ cells cultured in 200 $\mu$l serum-free RPMI-1640 medium were seeded onto Matrigel-coated 8-$\mu$m pore size Transwell filters (Corning Life Sciences, Corning, NY, USA) in the upper chambers. Five hundred microliters of RPMI-1640 containing 10% fetal calf serum was added to the lower chambers as a chemoattractant. Cells were incubated at 37˚C in a humidified 5% $CO_2$ atmosphere for 24 h. Cells that had successfully invaded through the inserts were fixed in 4% paraformaldehyde for 30 min, and stained with methylrosanilinium chloride. The invading cells were counted from five preselected microscopic fields of view at x200 magnification. The assay was performed from three independent experiments.

*Cell counting kit-8 (CCK-8) assay.* Cell proliferation was evaluated by CCK-8 assay (Beyotime Institute of Biotechnology). Cells were seeded in 96-well plates ($3x10^3$ per well). CCK-8 solution (10/100 $\mu$l medium) was added to each well, and cells were incubated for 1 h at 37˚C. Absorbance was measured at 450 nm using Synergy2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). The assay was conducted in five replicate wells for each sample and three parallel experiments were performed.

*Statistical analysis.* All statistical analyses were performed using SPSS version 17.0 (SPSS, Chicago, IL, USA). We used the $\chi^2$ test and Fisher's exact-test to analyze the correlations between PSD expression and clinicopathological characteristics. Student's t-test or one-way ANOVA were used for continuous variables. Data are expressed as mean ± SD. A value of $p<0.05$ was considered statistically significant.

## Results

*PSD expression is downregulated in GC cells and tissues.* Using real-time RT-PCR to assess mRNA expression of PSD, we found that PSD was downregulated in HGC27 (0.3651±0.020), AGS (0.4198±0.053), BGC823 (0.467±0.077) and SGC7901 (0.8188±0.009) cells compared to the normal mucosal line, GES1 (1-fold as the control) ($p<0.05$; Fig. 1A and B). We also analyzed PSD mRNA expression in 40 paired GC specimens and corresponding normal tissues. PSD mRNA expression was significantly lower in GC tissues than in their corresponding normal tissues (0.3406±0.017 vs. 0.5115±0.018), ($p<0.01$, Fig. 1C and D). Western blotting showed that protein expression of PSD in GC tissues was also lower than in their corresponding non-tumor tissues (0.2970±0.01706 vs. 0.5523±0.02593), ($p<0.01$, Fig. 2).

*Clinical significance of PSD protein expression in GC tissues.* We further investigated the relationship between PSD expression and clinicopathological factors. PSD mRNA and PSD protein levels were both related to tumor differentiation and

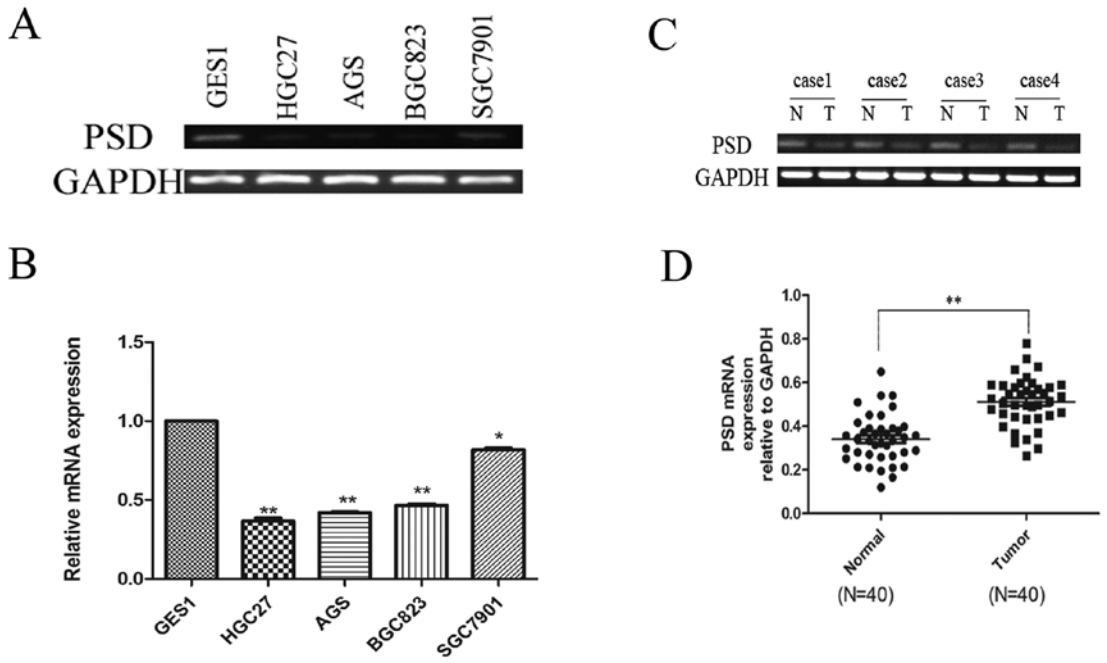


Figure 1. Real-time RT-PCR analysis of PSD mRNA expression in human GC cells and tissues. (A) Representative graphs of PCR assay in human GC cells. (B) Real-time RT-PCR analysis showed that expression of PSD in GES1 cells was higher than in the four different types of GC cell lines. (C) Representative graphs of PCR assay in human GC tissues. (D) Real-time RT-PCR analysis showed that PSD mRNA expression in GC tissues was significantly lower than in corresponding non-tumor tissues ($^{*}p<0.05$, $^{**}p<0.01$).

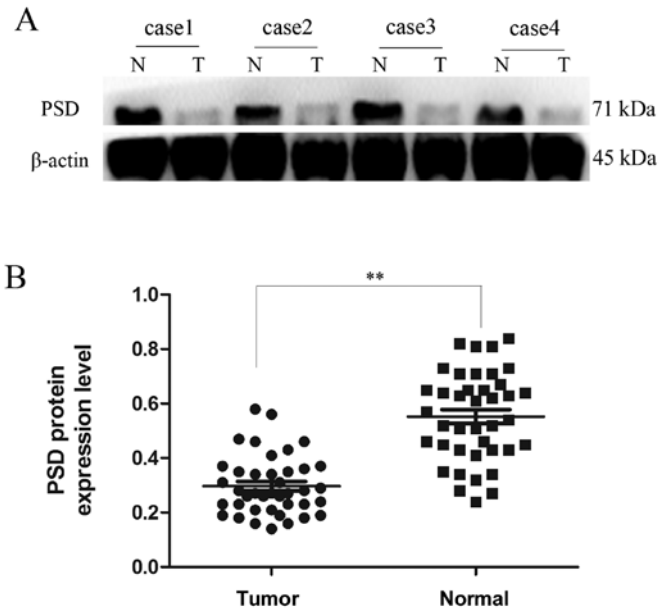


Figure 2. The protein expression of PSD was examined by western blotting. (A) Representative graphs of western blotting. (B) Quantitative analysis, relative levels of each protein compared to control β-actin were determined by densitometric scanning. Cases 1-4, sample number.

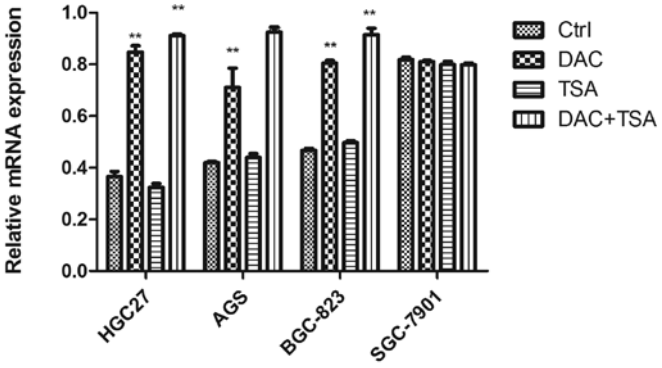


Figure 3. Real-time RT-PCR analysis of PSD mRNA expression after treatment with epigenetic agents ($^{**}p<0.01$).

lymph node metastasis (Tables I and II). There was no correlation with any of the clinicopathological features, including age, sex, tumor size, invasion depth and TNM stage.

*Abnormal histone modification is associated with PSD gene silencing in GC cell lines*. To elucidate whether the aberrant DNA methylation and histone modification of PSD were associated with loss of PSD, we treated GC cell lines HGC27, AGS, BCG823 and SGC7901 with epigenetic agents (DAC and TSA). DAC and TSA had different effects on PSD expression in the PSD-positive cell line (SGC7901) and PSD-negative cell lines (HGC27, AGS and BCG823). After treatment with DAC or TSA alone, the PSD mRNA was induced in HGC27, AGS and BCG823 cells. Combined treatment with both agents restored PSD expression to a significantly greater degree than did treatment with either agent alone. In SGC7901 cells, treatment with DAC and TSA, alone or in combination, had no significant effect on the restoration of PSD expression (Fig. 3).

To probe the role of histone modifications in the regulation of the PSD gene expression, we examined the histone markers H3-K9 trimethylation, H3-K9 acetylation and H3-K4 trimethylation with the PSD promoter region using ChIP. As shown

Table I. Correlation of PSD mRNA expression and clinicopathological parameters of gastric cancer samples.

| Variable | Patients (n), N=40 | PSD mRNA expression Low (%) | PSD mRNA expression High (%) | P-value |
|---|---|---|---|---|
| Age (years) | | | | |
| <65 | 18 | 15 (83.3) | 3 (16.7) | 0.289 |
| ≥65 | 22 | 17 (77.3) | 5 (22.7) | |
| Gender | | | | |
| Male | 21 | 17 (81.0) | 4 (19.0) | 0.724 |
| Female | 19 | 15 (78.9) | 4 (21.1) | |
| Tumor location | | | | |
| Upper + middle | 15 | 11 (73.3) | 4 (26.7) | 0.058 |
| Lower | 25 | 21 (84.0) | 4 (16.0) | |
| Tumor size (cm) | | | | |
| <3 | 22 | 17 (77.3) | 5 (22.7) | 0.289 |
| ≥3 | 18 | 15 (83.3) | 3 (16.7) | |
| Depth of invasion | | | | |
| T1+T2 | 17 | 14 (82.4) | 3 (17.6) | 0.480 |
| T3+T4 | 23 | 18 (78.3) | 5 (21.7) | |
| Differentiation | | | | |
| Well/moderate | 18 | 13 (72.2) | 5 (27.8) | 0.015[a] |
| Poor | 22 | 19 (86.4) | 3 (13.6) | |
| TNM stage | | | | |
| I+II | 15 | 12 (80.0) | 3 (20.0) | 1.000 |
| III+IV | 25 | 20 (80.0) | 5 (20.0) | |
| Lymph node metastasis | | | | |
| No | 18 | 12 (66.7) | 6 (33.3) | 0.000[a] |
| Yes | 22 | 20 (90.9) | 2 (9.1) | |

[a]P<0.01.

Table II. Correlation of PSD protein expression and clinicopathological parameters of gastric cancer samples.

| Variable | Patients (n), N=40 | PSD protein expression Low (%) | PSD protein expression High (%) | P-value |
|---|---|---|---|---|
| Age (years) | | | | |
| <65 | 18 | 14 (77.8) | 4 (22.2) | 0.665 |
| ≥65 | 22 | 14 (63.6) | 8 (36.4) | |
| Gender | | | | |
| Male | 21 | 15 (71.4) | 6 (28.6) | 0.645 |
| Female | 19 | 13 (68.4) | 6 (31.6) | |
| Tumor location | | | | |
| Upper + middle | 15 | 11 (73.3) | 4 (26.7) | 0.880 |
| Lower | 25 | 17 (68.0) | 8 (32.0) | |
| Tumor size (cm) | | | | |
| <3 | 22 | 16 (72.7) | 6 (27.3) | 0.874 |
| ≥3 | 18 | 12 (66.7) | 6 (33.3) | |
| Depth of invasion | | | | |
| T1+T2 | 17 | 11 (64.7) | 6 (35.3) | 0.635 |
| T3+T4 | 23 | 17 (73.9) | 6 (26.1) | |
| Differentiation | | | | |
| Well/moderate | 18 | 11 (61.1) | 7 (38.9) | 0.014[a] |
| Poor | 22 | 17 (77.3) | 5 (22.7) | |
| TNM stage | | | | |
| I+II | 15 | 10 (66.7) | 5 (33.3) | 0.443 |
| III+IV | 25 | 18 (72.0) | 7 (28.0) | |
| Lymph node metastasis | | | | |
| No | 18 | 10 (55.6) | 8 (44.4) | 0.000[a] |
| Yes | 22 | 18 (81.8) | 4 (18.2) | |

[a]P<0.01.

in Fig. 4, in SGC7901 cells, where PSD was expressed, the level of H3-K9 trimethylation in the promoter regions was minimal. In comparison, H3-K9 trimethylation levels in the PSD gene promoter were high in HGC27, AGS and BCG823 cells. We found that the levels of both H3-K9 acetylation and H3-K4 trimethylation at PSD promoter regions were minimal in HGC27, AGS and BCG823 cell lines. However, these levels were higher in SGC7901 cells. After treatment with DAC, H3-K9 trimethylation in the PSD promoter was decreased significantly and H3-K4 trimethylation was increased significantly in HGC27, AGS and BGC823 cells. We also found that TSA increased H3-K9 acetylation in the PSD promoter, but had no effect on H3-K9 trimethylation and H3-K4 trimethylation in HGC27, AGS and BGC823 cells. Combined treatment significantly decreased H3-K9 trimethylation, and increased H3-K9 acetylation and H3-K4 trimethylation in the PSD promoter in PSD-negative cell lines. The efficiency of combined treatment was similar to DAC or TSA alone. In the PSD-positive cell line (SGC7901), treatment with DAC, TSA or both had no significant effect on the histone markers H3-K9 trimethylation, H3-K9 acetylation and H3-K4 trimethylation.

*PSD gene silencing by DNA methylation in GC cell lines and primary GC*. The PSD promoters contain five CpG islands, so we can suppose that low PSD expression is due to DNA methylation. In order to test this hypothesis, we first examined the DNA methylation of PSD in GC cell lines using MSP.

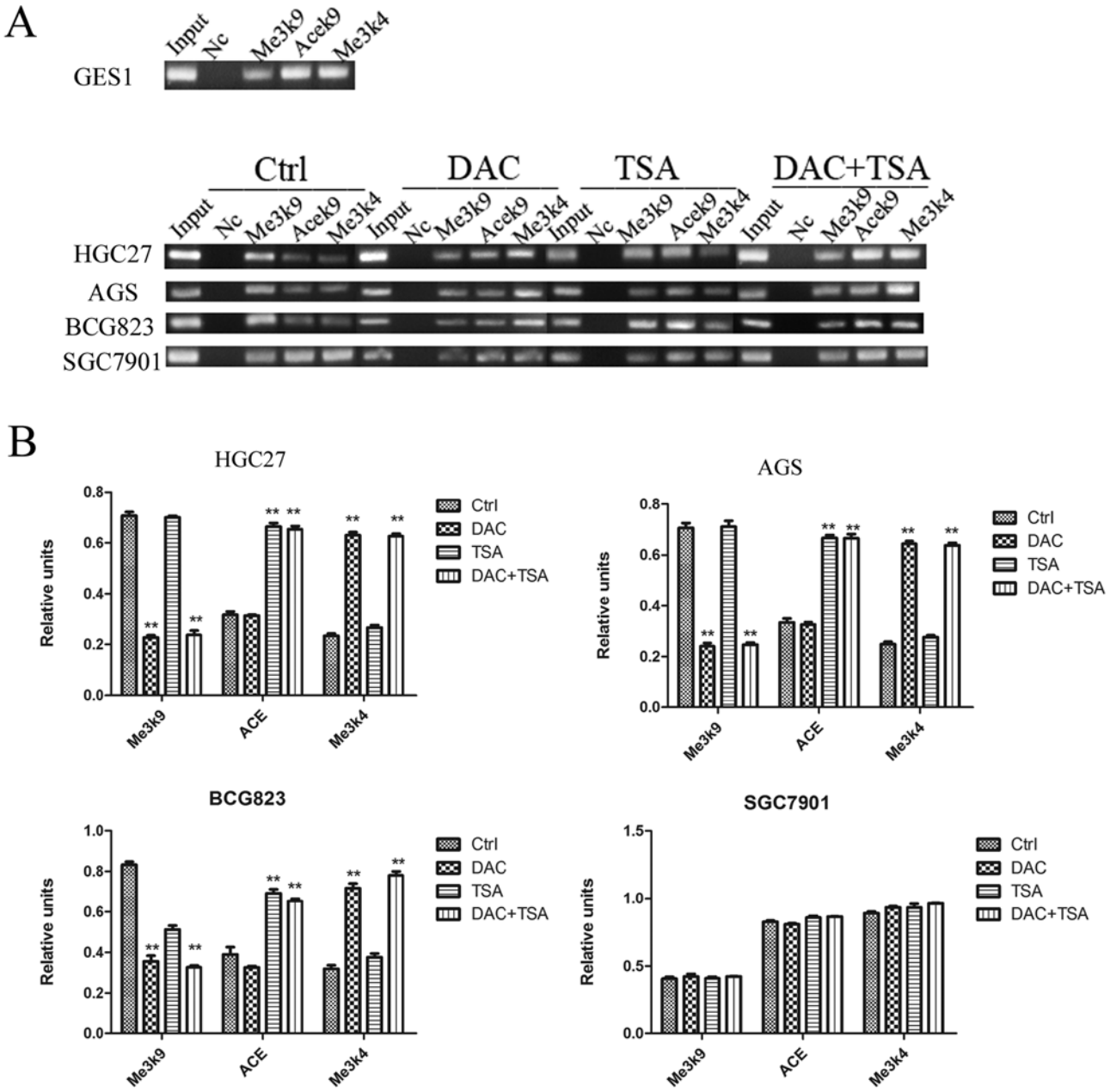


Figure 4. ChIP analysis of histone modification in the PSD promoter region in GC cell lines. Three independent ChIP assays were performed using an antibody that recognized H3-K9 trimethylation, H3-K9 acetylation and H3-K4 trimethylation at the PSD promoter region. (A) Representative graphs of ChIP-PCR assay. (B) Quantitative ChIP-PCR experiments were repeated three times. Average precipitated DNA/input DNA ratios shown on the y-axis represent the relative values of H3-K9 trimethylation, H3-K9 acetylation and H3-K4 trimethylation at the PSD promoter region ($^{**}p<0.01$).

We observed hypermethylation of PSD in HGC27 and AGS cells, partial methylation in BGC823, and no methylation in SGC7901 cells (Fig. 5). In PSD-negative cell lines (HGC27, AGS and BGC823), treatment with DAC resulted in DNA demethylation. TSA had no effect on DNA methylation of PSD, and combined treatment had no additional effect on DNA demethylation beyond that produced by treatment with DAC alone. In unmethylated SGC7901 cells, treatment with DAC, TSA or both did not have a significant effect on DNA methylation. MSP was performed to detect methylation status of PSD gene in 40 paired tumor and corresponding non-malignant gastric tissues. DNA methylation in gastric tissues, which included methylated and partially methylated tissues, occurred in 60% (24/40) of primary GC tissues and 27.5% (11/40) of non-malignant gastric tissues. No methylation was observed in 16 (40%) primary GC tissues and 29 (72.5%) non-malignant gastric tissues. The difference in methylation status of PSD between primary GC and non-malignant gastric tissue specimens was significant ($p<0.01$, Table III).

*Suppression of PSD expression improved migration and invasion of SGC7901 cells in vitro*. To explore further the tumor-suppressive function of PSD, we used PSD-specific siRNA to knock down PSD expression in the SGC7901 cell line, in which the level of PSD was relatively high. RT-PCR confirmed that PSD was downregulated after 48-h transfection (Fig. 6A). Wound-healing assay showed that in SGC7901 cells, knockdown of PSD led to cell migration at 24 h after

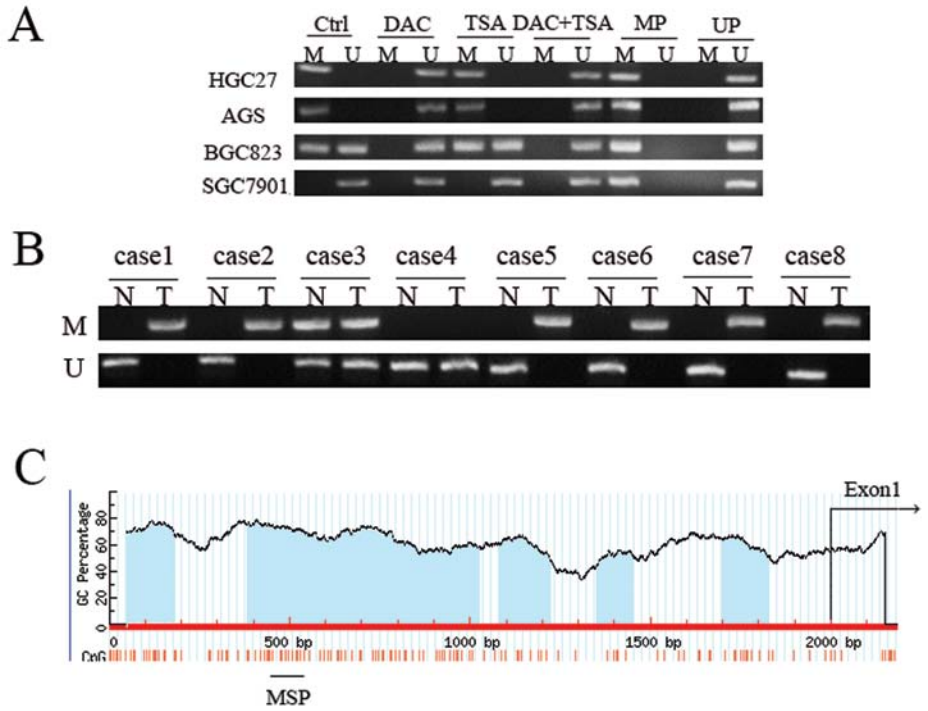


Figure 5. MSP analysis of DNA methylation of PSD gene in human GC cells and tissues. At least three independent experiments were performed with similar results. (A) MSP analysis of DNA methylation at the PSD promoter region before and after treatment with DAC, TSA or both. (B) DNA methylation of PSD in GC specimens and corresponding non-malignant gastric tissues. (C) Graphic representation of CpG islands in PSD promoter region. Lane M, the presence of methylated alleles. Lane U, the presence of unmethylated alleles. UP, non-methylation positive control; MP, methylation positive control; N, non-malignant gastric tissue; T, tumor specimens. Cases 1-8, sample number.

Table III. Methylation status of PSD between gastric tissues (T) and non-malignant gastric tissues (N).

| Group | Case | Methylation (%) | No methylation (%) | P-value |
|---|---|---|---|---|
| T | 40 | 24 (60.00) | 16 (40.00) | <0.01[a] |
| N | 40 | 11 (27.50) | 29 (72.50) | |

[a]P<0.01.

establishing the wound (Fig. 6B). We also examined whether cell invasive capacity was altered in PSD-depleted cells. Matrigel invasion assay showed that SGC7901 cells transfected with PSD siRNA had more invasion (187.5±4.518) than cells transfected with control siRNA (66.5±1.848) or untreated SGC7901 cells (64.2±1.250) ($p<0.01$, Fig. 6C).

*Depletion of PSD reduces apoptosis of SGC7901 cells*. To evaluate the potential effects of transfection with PSD siRNA on growth of SGC7901 cells, we examined the growth curve by CCK-8 assay. Depletion of PSD promoted growth of SGC7901 cells in a time-dependent manner (Fig. 7A). We then sought to determine the possible mechanisms that underlie the depletion of PSD-promoted growth of SGC7901 cells. Levels of apoptosis-related proteins (caspase-3 and -7) were evaluated using western blot analysis. Caspase-3 and -7 protein levels decreased after 48 h of transfection with PSD siRNA (Fig. 7B).

## Discussion

We found that PSD expression was significantly reduced in GC cell lines and tissues compared to GES1 and normal tissues. PSD mRNA expression in GC tissues was related to tumor differentiation and lymph node metastasis. This is in agreement with the result that differential PSD expression in the GC cell lines may be related to cell differentiation. PSD mRNA expression in human poorly differentiated gastric carcinoma cell line HGC27 was lower than in human moderately differentiated gastric carcinoma cell line SGC7901. This suggests that the degree of malignancy of GC may be higher when the expression of PSD is low.

We also identified three mechanisms underlying the decreased expression of PSD: DNA hypermethylation of PSD promoter, hypertrimethylation of histones H3-K9, and hypotrimethylation of histones H3-K4 attached to the promoter. First, using ChIP techniques in four GC cell lines and GES-1 cell line, we showed that the level of H3-K9 trimethylation in the promoter regions of PSD-negative cell lines (HGC27, AGS and BCG823) was higher than in GES-1 and PSD-positive cell line SGC7901. In contrast, the level of H3-K9 acetylation and H3-K4 trimethylation in the PSD promoter region was lower than in GES-1 and PSD-positive cell line SGC7901. Second, we examined the DNA methylation of PSD in GC cell lines and tissues by MSP. The level of DNA methylation in the promoter regions of PSD-negative cell lines (HGC27, AGS and BCG823) was higher than in PSD-positive cell line SGC7901. Lastly, we treated the GC cell lines with DAC and TSA and found that DAC or combined treatment restored PSD expres-

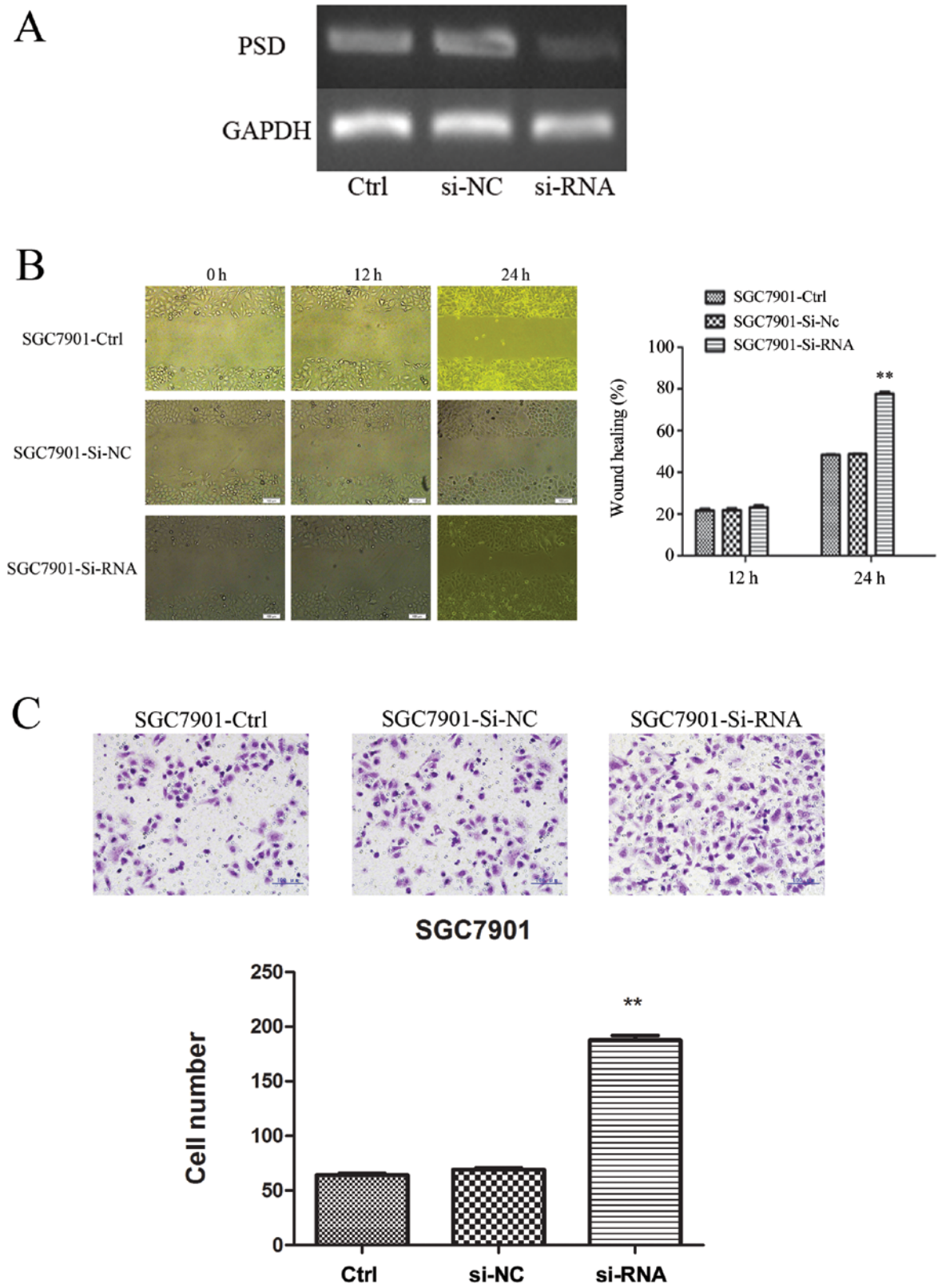


Figure 6. Migration and invasion enhancement of SGC7901 cells after knocking down PSD expression. (A) Evaluation of PSD expression after transfection with siRNA-PSD, using RT-PCR. (B) Wound healing assay of SGC7901 cells. Images were taken at 0, 12 and 24 h after the wound was made (x100). Cell migration was enhanced at 24 h after knocking down PSD. (C) Matrigel invasion assay showed that knocking down PSD in SGC7901 cells significantly increased cell invasiveness.

sion to a significantly greater degree by means of reversing the level of H3-K9 trimethylation, H3-K4 trimethylation and DNA hypermethylation. TSA significantly increased H3-K9 acetylation but the effect on restored PSD expression was limited. Thus, we have reason to believe that silencing of PSD genes is mainly due to H3-K9 trimethylation, H3-K4 trimethylation

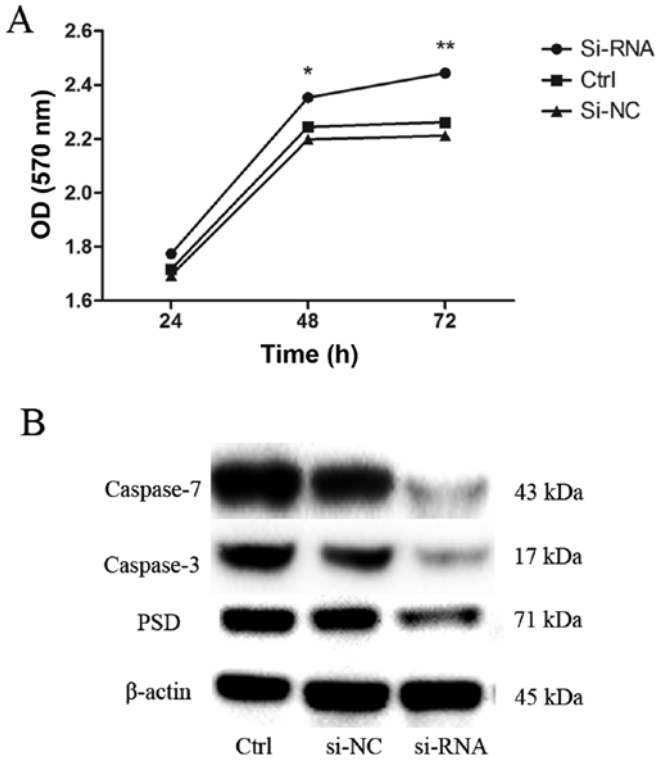


Figure 7. Inhibition of PSD reducing apoptosis of SGC7901 cells. (A) CCK-8 assay showed that proliferation of SGC7901 cells was significantly promoted at 48 and 72 h after knocking down PSD. (B) Western blot analysis of PSD, caspase-3 and -7 protein levels at 48 h after transfection with PSD siRNA. β-actin was used as the loading control.

and DNA hypermethylation but not H3-K9 acetylation. Our results are consistent with a model in which H3-K9 methylation and DNA methylation closely collaborate in maintaining the repressive state of tumor TSGs. H3-K4 trimethylation is associated with an open chromatin configuration and activation of TSG transcription. H3-K4 trimethylation in promoter regions of PSD was inversely correlated with DNA methylation status (22-24).

To investigate further the tumor-suppressive functions of PSD, SGC7901 cells were treated with PSD-specific siRNA to knock down PSD expression. Cell migration and invasion were markedly enhanced when PSD was silenced. These results are in agreement with our present study that the expression of PSD was statistically significantly inverse correlated with lymph node metastasis. This provides further evidence that PSD may function as a tumor suppressorin gastric cancer.

We also appraised the effect of PSD-specific siRNA on SGC7901 cell growth, which indicated that depletion of PSD promoted growth of SGC7901 cells. Next, we assessed the levels of apoptosis-related proteins caspase-3 and -7, and found that they decreased after 48-h transfection with PSD siRNA. These findings were also supported by a similar study in a human promyelocytic leukemia cell line, HL-60 (25). The mechanism underlying modifications of depletion of PSD reducing apoptosis is not clear. One possibility is that Fas-induced apoptosis is mediated by the activation of a Ras-related C3 botulinum toxin substrate 1 (Rac1), and PSD can regulate Rac1 (26,27). Rac1 plays a pivotal role in inducing apoptosis in response to stimuli such as UV light (28), tumor necrosis factor-α (16) and Fas (15). These findings strongly support our data showing that PSD silencing can inhibited Rac1-mediated apoptosis in siPSD-treated SGC7901 cells.

This is believed to be the first report of the tumor-suppressor functions of PSD, which are epigenetically silenced in GC, while silencing of PSD after transfection with siRNA promotes GC progression. These findings provide new targets for prognosis and pharmacological intervention in human GC. Therefore, further *in vitro* and *in vivo* studies are needed to determine the precise mechanism of PSD in the progression of GC.

## Acknowledgements

This study was supported in part by a grant from the National Natural Science Foundation of China (grant no. 30572162), the Foundation of Liaoning Province Science and Technology Plan Project (grant no. 2013225021) and the Higher Specialized Research Fund for Doctoral Program of Ministry of Education of China (grant no. 20102104110001).

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