

Psoriasin overexpression confers drug resistance to cisplatin by activating ERK in gastric cancer

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Abstract. Psoriasin, a member of the S100 multigenic family, which is aberrantly expressed in a variety of human tumors, is considered as an attractive molecular target for cancer treatment. The present study aimed to characterize the role of psoriasin in gastric cancer (GC), the associated pathways through which it contributes to cancer development and progression, and the effect of psoriasin on cellular response to pre-operative chemotherapy in patients with GC. Expression of psoriasin mRNA and protein were analyzed using quantitative polymerase chain reaction and immunohistochemistry of gastric cancer cohorts, respectively. Gastric cancer cell models with differential expression of psoriasin were generated using stable cell lines that overexpressed psoriasin. The *in vitro* biological functions of the cells in response to psoriasin overexpression and to chemotherapeutic agents were assessed using various cell-based assays. Psoriasin was overexpressed in patients with advanced GC, and high psoriasin levels led to poor clinical outcomes. Increasing psoriasin expression in GC cell lines promoted cell proliferation, migration and invasion *in vitro*. Furthermore, psoriasin overexpression caused alterations in the levels of epithelial-mesenchymal transition-associated proteins, and activated the extracellular signal-regulated kinase signaling pathway. Additionally, higher levels of psoriasin expression were significantly associated a lack of response to neoadjuvant chemotherapy in patients with GC. Psoriasin overexpression tended to decrease the sensitivity

of GC cells to cisplatin, potentially by inhibiting apoptosis or increasing the S-phase population. Taken together, these results indicate that psoriasin may be a promising therapeutic target for GC treatment, and a potential molecular marker to predict patient response to pre-operative chemotherapy.

Introduction

Gastric cancer (GC) currently ranks as the fourth most frequently diagnosed cancer and the second leading cause of mortality from malignant diseases worldwide (1). According to statistics, >950 000 new GC cases are diagnosed every year, and an estimated 723,100 mortalities occurred in 2012 (1,2). By the time of diagnosis, the majority of patients present with advanced GC, with a 5-year survival rate of <30% (3,4). Pre-operative chemotherapy has been successfully used in the treatment of locally advanced GC, as it may increase the possibility of complete resection through shrinking the tumor (5). Based on clinical data, patients with GC that exhibit a positive response to neo-adjuvant chemotherapy can obtain a better prognosis than non-responders (5). However, different patients exhibit distinct therapeutic responses due to various genetic and epigenetic alterations that are involved in gastric tumorigenesis (4,6). Therefore, a comprehensive understanding of the molecular variables that affect the GC disease pathway may contribute to identifying novel therapeutic targets or prognostic biomarkers that may help identify patients that are likely to benefit from chemotherapy.

Psoriasin, also termed S100A7, belongs to the S100 multigenic family of calcium-binding EF-hand proteins and was initially identified in 1991 as a highly expressed protein in psoriatic keratinocytes (7,8). Psoriasin, a low molecular weight protein (11.4 kDa), is localized in the cytoplasm, cell nucleus or secreted outside of the cell in certain cases (9,10). Dysregulated expression of psoriasin has been observed in various types of cancer, including oral squamous cell carcinoma (11), breast cancer (10,12), lung cancer (13), bladder cancer (14), skin cancer (15), head and neck cancer (16), prostate cancer (17) and cervical cancer (18). Functionally, psoriasin usually has malignant functions in cancer; however, it can also exhibit tumor suppressive effects in certain types of oral carcinoma and breast cancer by decreasing β -catenin activity (11,12). Subsequent studies have suggested that intracellular and secreted forms of psoriasin protein have an important role in tumor development

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by regulating cell proliferation, metastasis, migration, invasion, apoptosis and angiogenesis (17-19). Mechanistically, several signaling pathways have been demonstrated to have a correlation with psoriasin, including Hippo, receptor for advanced glycation endproducts (RAGE), extracellular signal-regulated kinase (ERK), β -catenin and nuclear factor (NF)- κ B pathways. The downregulation of psoriasin in lung cancer suppresses cell growth and invasive potential by regulating NF- κ B activity and the expression of matrix metalloproteinases and vascular endothelial growth factor (19). Similarly, psoriasin promotes cell survival by binding to c-Jun activation domain-binding protein1 and enhancing the phosphorylation of NF- κ B and protein kinase B in breast cancer (20). Another study demonstrated that psoriasin binds to RAGE and activates ERK signaling to increase migration and invasion in cervical cancer (18).

However, the role of psoriasin in GC and its underlying mechanisms remain unclear. To the best of our knowledge, the present study is the first to characterize the expression of psoriasin in patients with GC and investigate the role of psoriasin in GC pathogenesis. Psoriasin was markedly upregulated in patients with GC and high expression of psoriasin was associated with poor survival. Furthermore, psoriasin positively regulated ERK signaling and promoted proliferation, migration and invasion. In addition, overexpression of psoriasin decreased the sensitivity of GC cells to cisplatin. Taken together, the findings suggested that psoriasin has a critical role in the pathogenesis of GC and may be a novel therapeutic target or a potential diagnostic marker for the patient response to pre-operative chemotherapy in GC.

Materials and methods

Cell line and cell culture. The human AGS (no. 89090402) and HGC-27 (no. 94042256) GC cell lines were purchased from the European Collection of Animal Cell Cultures (ECACC; Salisbury, UK). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich; Merck KGaA) and 1% antibiotics (penicillin and streptomycin; Sigma-Aldrich; Merck KGaA) at 37°C in a humidified 5% CO₂ atmosphere.

Patients and human GC tissue. Two cohorts were used in the present study, namely a generic collection and a collection with chemosensitive information. The first cohort had a total of 321 GC tissues and 173 adjacent normal tissues were collected from patients with GC that underwent surgical resection, and some were treated with preoperative radiotherapy or chemotherapy at the Peking University Cancer Hospital (Beijing, China). Tissue and data were obtained between September 2003 and December 2007. The mean age of the patients in the cohort was 59.8 years with male/female ratio of 229:92 (Table I). The other cohort comprised of 87 patients with GC that had received chemotherapy with oxaliplatin/5-fluorouracil/leucovorin prior to surgery at the Peking University Cancer Hospital between December 2001 and November 2006. All of the tissues were stored immediately following surgery at -80°C until use. Clinicopathological and follow-up information was obtained from patient data. GC staging was determined according to the 2010 tumor-node-metastasis (TNM) classification recommended by the American

Table I. Association between psoriasin mRNA expression and clinicopathological features in gastric cancer tissues.

Variable	n	Psoriasin expression (median)	P-value
Tissue			<0.001
Tumor	321	12.00	
Adjacent normal	173	0.04	
Sex			0.573
Male	229	9.00	
Female	92	13.00	
Depth of invasion			0.011
T1-2	42	1.00	
T3-4	279	15.00	
Lymph node status			<0.001
N0	71	0.03	
N1+2+3	250	16.00	
Distant metastasis			<0.001
M0	280	4.00	
M1	41	384.00	
TNM staging			<0.001
I+II	85	1.00	
III+IV	236	17.00	
Differentiation			
High	1	0.15	
High-medium	21	20.00	
Medium	67	15.00	0.629 ^a
Medium-low	87	13.00	0.732 ^a
Low	145	3.00	0.453 ^a
Clinical outcome			<0.001
Alive	134	1.00	
Died	187	25.00	

^aCompared with high-medium differentiation; Mann-Whitney U test for comparisons between two groups; Kruskal-Wallis test for multiple group followed Tukey honest significant difference as post hoc test. TNM, tumor-node-metastasis.

Joint Committee on Cancer (AJCC 7th edition) (21). This study was approved by Peking University Cancer Hospital Research Ethics Committee (ethics no. 2006021) and written consent was obtained from all patients involved.

RNA isolation, reverse transcription, conventional polymerase chain reaction (PCR) and quantitative PCR (qPCR). Total RNA was extracted from cells or homogenized tissues using TRI reagent (Sigma-Aldrich; Merck KGaA) following the manufacturer's instructions, and subsequently reverse transcribed into cDNA using Promega Reverse Transcription kit (Promega Corporation, Madison, WI, USA). The reverse transcription conditions were annealing at 25°C for 5 min, extension at 42°C for 60 min, and inactivation at 70°C for 15. PCR was then performed using GoTaq Green MasterMix (Promega Corporation) under the cycling conditions as follows:

Table II. Primers sequences.

Name	Forward	Reverse
Psoriasin	ATGAGCAACACTCAAGCTG	ACTGGCTGCCCCCGGAACA
Psoriasin (qPCR)	TGTGACAAAAAGGGCACAAA	<u>ACTGAACCTGACCGTACAC</u> CCAGCAAGGACAGAACTC
Snail	CGCTCTTTCTCTCGTCAG	GTTGCAGTATTTGCAGTTGA
Slug	CTCTCTCTTTCCGGATACT	AGCAGTTTTTGCAGTGGTAT
Vimentin	GATGCTTCAGAGAGAGGAAG	CTCTTCGTGGAGTTTCTTCA
E-cadherin	CGAGAGCTACACGTTTCAC	GGGAAAAATAGGCTGTCTCT
N-cadherin	CAACGACGGGTAGTCAC	ATTGGGGTCTGGAGTTTC
Zeb-1	CTTGTGATTTGTGTGACAAGA	ATGCCTTTTTACAGATTCCA
MMP-2	TTTGATGACGATGAGCTATG	TGCAGCTCTCATATTTGTTG
MMP-3	TCATTTTGGCCATCTCTTCC	GTGCCCATATTGTGCCTTCT
MMP-9	AACACGACCGGGACAAG	ATTCACGTCGTCCTTATGC
ERK1	ACACGCAGTTGCAGTACA	CCACATACTCCGTCAGGA
ERK2	CCAACCTCTCGTACATCG	GTCAGGAACCCTGTGTGA
c-Fos	CAGACTACGAGGCGTCATCC	TCTGCGGGTGAGTGGTAGTA
c-Jun	AAGATCCTGAAACAGAGCAT	GCTGGACTGGATTATCAGG
Cyclin D1	CGGTGTCCTACTTCAAATGT	ACCTCCTCCTCCTCCTCT
Cyclin D2	GGAGAAGCTGTCTCTGATCC	GGGTACATGGCAAACCTAAA
GAPDH	CGCTGCTTTTAACTCTGGTA	GACTGTGGTCAGAGTCCTT
GAPDH (qPCR)	CTGAGTACGTCGTCGTGGAGTC	<u>ACTGAACCTGACCGTACAC</u> AGAGATGATGACCCTTTTG
Actin (qPCR)	GGACCTGACTGACTACCTCA	<u>ACTGAACCTGACCGTACA</u> AGCTTCTCCTTAATGTCACG

The underlined sequence in the reverse primers is the additional Z sequence which is complementary to the universal Z probe. qPCR, quantitative polymerase chain reaction; Zeb-1, zinc finger E-box-binding homeobox 1; MMP, matrix metalloproteinase; ERK, extracellular signal-regulated kinase.

95°C for 2 min, followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, and a final extension of 5 min at 72°C. The PCR products were visualized on 1-2% agarose gels stained with SYBR Safe (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The primer sequences used for PCR in this study are listed in Table II.

qPCR was run on the StepOne Plus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.), using Precision FAST 2X qPCR MasterMix (Primerdesign Ltd., Chandler's Ford, UK) in a final volume of 10 µl. cDNA samples from GC cells and two cohorts of GC tissues were examined for psoriasin transcript expression with standard and negative controls. Reaction conditions were as follows: 94°C for 10 min, followed by 100 cycles of 94°C for 10 sec, 55°C for 30 sec and 72°C for 10 sec. GAPDH or actin was used as an internal control. The primer sequences used for qPCR are listed in Table II. Primers for qPCR were designed to the specific target using the Beacon Designer software (version 2; Premier Biosoft International, Palo Alto, CA, USA). An additional sequence, known as the Z sequence (5'-ACTGAACCTGACCGTACA-3'), which acts as a template for the amplifluor uniprimer probe, was added with the reverse primers. Expression of the target sequence was qualified in conjunction with a range of known dilution series of podoplanin gene transcript, which are used to generate a standard curve to enable the calculation of transcript copy.

Construction of psoriasin overexpression vector in GC cell lines. Full-length human psoriasin coding sequence was

amplified from human prostate tissues with appropriate primers (Table I). The synthesized product was cloned into the pEF6/V5-His-TOPO plasmid vector (K961020; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The overexpression constructs and control plasmids (25 µg pre transfection) were then transfected into 2.5x10⁶ AGS and HGC-27 cells using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After 2 weeks of selection using 6 µg/ml blasticidin (Melford Laboratories, Ltd., Ipswich, UK), stable transfectants were obtained and verified for psoriasin overexpression. Then cells were cultured in DMEM with a lower concentration of blasticidin (0.6 µg/ml) to maintain plasmid expression.

Treatment with ERK inhibitor and cisplatin. AGS and HGC-27 were cultured in serum-free medium for 4-6 h, and then treated with 1 µM ERK inhibitor (FR 180204; Tocris Bioscience, Bristol, UK) or 16.6 µM cisplatin (Tocris Bioscience) for 24 h, followed by PCR, western blotting or flow cytometry analysis.

Western blotting. AGS and HGC-27 were isolated by pre-chilled lysis buffer (50 mM Tris-Cl, pH 7.5, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl) containing protease inhibitor cocktail (aprotinin and leupeptin) and phosphorylation inhibitor (sodium vanadate) and qualified with a DCTM protein assay kit (Bio-Rad Laboratories, Inc.). Protein extract of each sample (30 µg) was separated by 10% or 12% SDS-PAGE and transferred onto a 0.45 µm

polyvinylidenedifluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat dried milk in PBST solution (0.05% Tween-20 in PBS) for 1 h at room temperature. The blocked membranes were then incubated in a diluted primary antibodies (1:500) overnight at 4°C, washed in PBST and incubated with a diluted specific horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000) for 1 h at room temperature. The following primary antibodies were used in the study: Anti-S100A7/psoriasin (clone 47C1068; catalog no.; Novus Biologicals, Ltd., Cambridge, UK); phospho (p)-ERK (Tyr 204; cat. no. sc-7976), ERK1/2 (clone C-9; cat. no. sc-514302), p21 (cat. no. sc-6246), p53 (clone DO-1; cat. no. sc-126), vimentin (cat. no. sc-66002), E-cadherin (cat. no. sc-1500), N-cadherin (cat. no. sc-7939), zinc finger E-box binding homeobox 1 (Zeb-1; cat. no. sc-81428) and GAPDH (cat. no. sc-32233; all Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The HRP secondary antibodies (cat. no. A5278, anti-mouse IgG; cat. no. A0545, anti-rabbit IgG) were obtained from Sigma-Aldrich (Merck, KGaA). Detection was performed using an EZ-ECL chemiluminescence kit (Biological Industries USA, Inc., Cromwell, CT, USA). Immunoreactive bands were visualized and quantified by densitometry using the Syngene G:BOX chemiluminescence imaging system and Gene Tools 4.03 (Syngene Europe, Cambridge, UK).

Cell proliferation assay. Briefly, AGS and HGC-27 cells were seeded into 96-well plate (2×10^3 cells/well) in 6 replicates followed by incubation for 0, 1, 2, 3, 4 and 5 days. Following fixation in 4% formalin for 30 min at room temperature, the cells were stained with 0.5% (w/v) crystal violet (Sigma-Aldrich) for 15 min at room temperature. Following washing, the cells were then extracted with 10% (v/v) acetic acid and cell density was determined by measuring the absorbance at 540 nm using a spectrophotometer (Elx800; BioTek Instruments, Inc., Winooski, VT, USA).

Cell viability assay. To evaluate the cytotoxicity of cisplatin, AGS and HGC-27 cells were plated into 96-well plate (cells/well) in 6 replicates. After 24-h incubation, the cells were exposed to serial concentrations of cisplatin (0, 3.125, 6.25, 12.5, 25 and 50 μ M, respectively) for 72 h. Cells viability was assessed by staining with 0.5% (w/v) crystal violet for 15 min at room temperature.

Cell colony assay. AGS and HGC-27 were plated into 6-well plate at the density of 400 cells/ well and incubated at 37°C and at an atmosphere of 5% CO₂ for 2 weeks. The cell colonies were fixed in 4% formalin for 30 min and stained with 0.5% (w/v) crystal violet for 15 min. After images were taken with a camera, the colonies were dissolved in 10% (v/v) acetic acid, and the absorbance was measured at 540 nm using a spectrophotometer (Elx800; BioTek Instruments, Inc.).

Cell-matrix adhesion assay. A total of 2×10^4 cells were plated in each well of a black 96-well plate previously coated with Matrigel (10 μ g/well; Corning Incorporated, Corning, NY, USA) in 6 replicates. After incubation for 1 h, the cells were washed once with PBS and then incubated in cell dissociation solution (100 μ l/well; Sigma-Aldrich; Merck KGaA) for 30 min containing 0.002 μ g/ μ l calcein AM (eBioscience; Thermo Fisher Scientific, Inc.). Fluorescence of the cell suspension was

read using a GloMax®-Multi Detection System (Promega Corporation) at 495 nm for excitation and 519 nm for emission.

In vitro invasion assay. Cells (5×10^4) were loaded into an 8 μ m-pore ThinCert™ 24-well plate insert (Greiner Bio-One International GmbH, Kremsmünster, Austria) coated with 100 μ g Matrigel (each sample in triplicate). The upper chamber was provided with serum-free medium, and the lower chamber was filled with DMEM with 10% FCS. Following incubation for 24 h, the underside of the insert was incubated in 350 μ l dissociation solution containing 1 μ M calcein AM for 1 h. The cell suspension was then aliquoted into a black 96-well plate (100 μ l/well) and read using the GloMax®-Multi Detection System at 495 nm for excitation and 519 nm for emission.

In vitro cell migration assay and the effect of ERK inhibitor on cell mobility. Electric cell-substrate impedance sensing (ECIS) instruments (Applied Biophysics, Inc., Troy, NY, USA) were used to monitor cell migration by detecting impedance variation based on the current flow change on the gold electrodes of a 96-well array (Applied Biophysics Inc.) (22). There are two parameters, resistance and capacitance, derived from current and voltage measurements. Among them, resistance, consisting of para- and trans-cellular current flow, is better to represent the quality and function of cell barrier (23). Briefly, 6×10^4 cells diluted in 200 μ l DMEM with or without ERK inhibitor (1 μ M) were seeded into each ECIS 96-plate well, and the cells were wounded by applying electric current (3,000 μ A, 60 kHz) when confluent monolayers were formed. The array was placed into a CO₂ incubator, which was connected to the ECIS Model 9600 Controller, and the impedance data, which indicate cellular migration, were collected continuously for 6 h. In this study, the normalized resistance measured at 4,000 Hz was chosen to evaluate cell function due to its high sensitivity to the current flow variation caused by the change of cell behavior as described in the literature (24).

Flow cytometric analysis. For protein expression analysis, AGS and HGC-27 were fixed with IC Fixation Buffer (eBioscience; Thermo Fisher Scientific, Inc.) at room temperature for 30 min, and then permeabilized in ice-cold 100% methanol at 4°C for 30 min. Following blocking with PBS containing 0.1% Tween-20 (v/v) and 1% bovine serum albumin (w/v) (Sigma-Aldrich; Merck KGaA), the cells were incubated with a cleaved poly(ADP-ribose) polymerase 1 (PARP1) antibody (1:40; cat. no. 44-698G; eBioscience; Thermo Fisher Scientific, Inc.) overnight at 4°C. Mouse IgG2b K isotype antibody (eBioscience; Thermo Fisher Scientific, Inc.) was used as a control. The samples were then washed by PBS twice and resuspended in PBS containing 2 mM EDTA and 5% FCS for flow cytometry.

For cell cycle analysis, the cells were fixed with 70% ethanol for 15 min on ice, and stained with FxCycle™ PI/RNase staining solution (Thermo Fisher Scientific, Inc.) for 30 min. For apoptosis analysis, the cells were stained using an Annexin V Apoptosis Detection kit (eBioscience; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Flow cytometry was performed using a Canto II flow cytometer (BD Bioscience, San Jose, CA, USA), and the data were analyzed using FCS Express version 4 Research Edition (De Novo Software, Glendale, CA, USA).

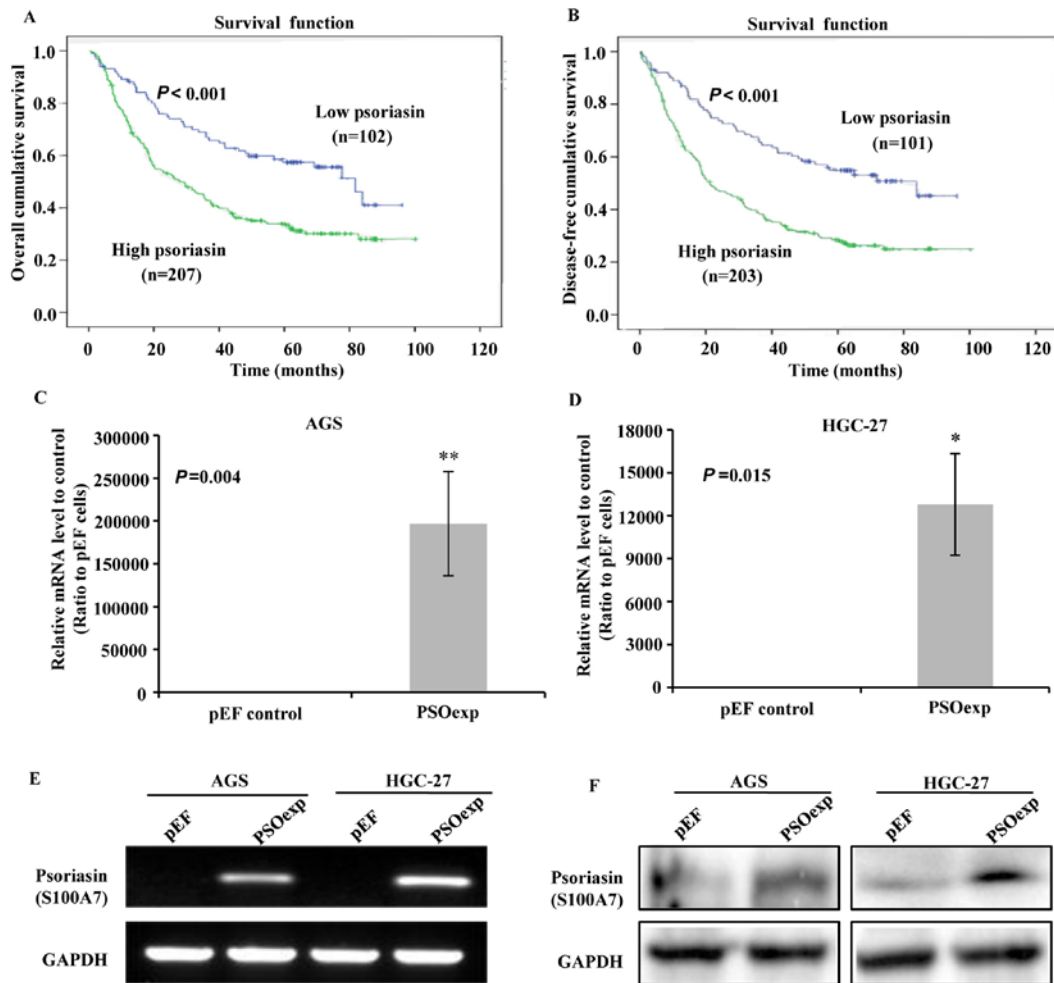


Figure 1. Survival curves of patients with GC, and the validation of psoriasin overexpression in GC cell lines. The high-low groups of patients were divided the median transcript values of psoriasin, guided by clinical stages (TNM staging). (A) Association between the transcript levels of psoriasin and overall survival. Low psoriasin group (median expression, 1), n=102; high psoriasin expression (median expression, 25), n=207, $P < 0.001$. (B) The correlation between the transcript levels of psoriasin and disease-free survival. Low psoriasin group (median expression, 1), n=101; high psoriasin expression (median expression, 25), n=203, $P < 0.001$. Reverse transcription-quantitative PCR analyzing mRNA expression of psoriasin in (C) AGS and (D) HGC-27 cell line, which was normalized with the reference gene GAPDH. The data are presented as a ratio to pEF control. (E) Confirmation of psoriasin overexpression in AGS and HGC-27 cells using conventional PCR. (F) Western blot analysis of the level of psoriasin expression in AGS and HGC-27 cell lines using an anti-S100A7/psoriasin antibody (clone 47C1068). * $P < 0.05$, ** $P < 0.01$ vs. pEF control. PCR, polymerase chain reaction; pEF, cells transfected with the empty plasmid vector; PSOexp, cells transfected with psoriasin plasmid.

Statistical analysis. The results are presented as data from three independent experiments and expressed as the mean \pm standard deviation. The cumulative survival curves were generated using Kaplan-Meier plots and analyzed using log-rank test. Kruskal-Wallis test was used for comparisons of three or more groups followed by Tukey Honest Significant Difference as a post hoc test, and Mann-Whitney U test was used for comparisons between two groups. Statistical analysis was performed using SPSS v18 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of psoriasin transcripts and its correlation with clinicopathological parameters in GC tissues. To determine the clinical relevance of psoriasin in GC progression, the transcript levels of psoriasin in GC (n=321) and the adjacent normal specimens (n=173) were examined by RT-qPCR. As presented in in

Table II, psoriasin was higher in GC tissues than in adjacent normal tissues ($P < 0.001$), and a higher level of psoriasin was observed in advanced stages of GC (TNM III+IV) compared with early stages (TNM I+II; $P < 0.001$; Table II). Notably, higher expression of psoriasin was detected in tumors with deeper infiltration (T3-4 vs. T1-2; $P = 0.011$), lymph node involvement ($P < 0.001$) and distance metastasis ($P < 0.001$, Table II). Furthermore, psoriasin exhibited higher expression in patients with GC that had died prior to the end of follow-up than in those who remained alive at the end of the follow-up period ($P < 0.001$; Table II). Kaplan-Meier analysis and log-rank test revealed that patients with GC with higher expression of psoriasin had worse overall survival ($P < 0.001$; Fig. 1A) and disease-free survival ($P < 0.001$; Fig. 1B), compared with those with lower expression.

Psoriasin overexpression promotes cell proliferation, invasion and migration in AGS and HGC-27, and decreases adhesion in HGC-27 cells. AGS and HGC27 are two well-characterized gastric cancer cell lines (25,26). As demonstrated in Fig. 1,

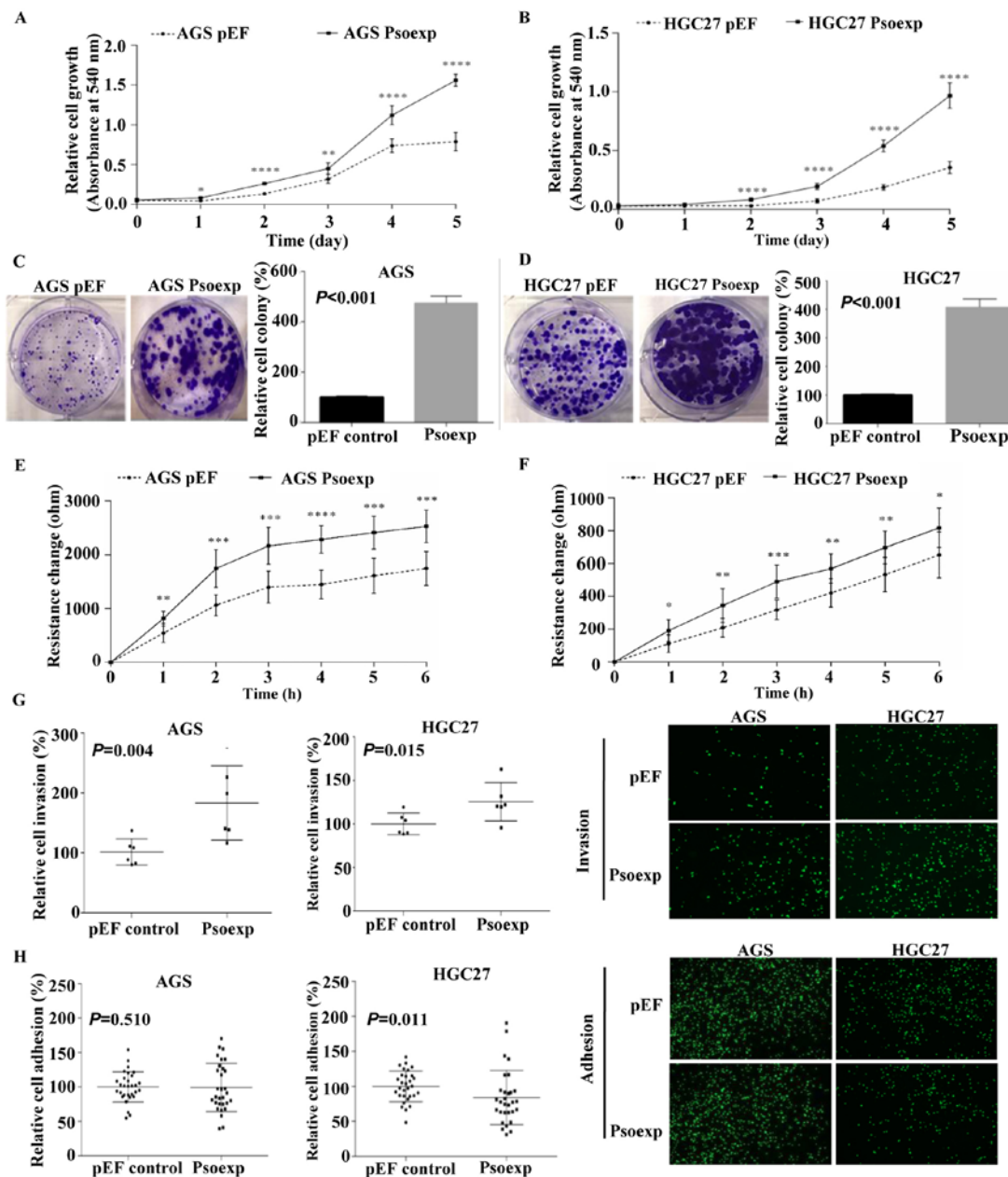


Figure 2. Effect of psoriasin overexpression on the functions of AGS and HGC-27 cell lines *in vitro*. (A) Growth of cells that overexpress psoriasin (AGS Psoexp) compared with its control (AGS pEF). (B) Growth of cells that overexpress psoriasin (HGC-27 Psoexp) compared with its control (HGC-27 pEF). (C) Colony-formation ability in AGS cells following psoriasin upregulation. (D) Increase in colony-formation ability in HGC-27 cell following psoriasin upregulation. Migration of (E) AGS and (F) HGC-27 cells determined by the ECIS system. Data are presents as resistance change (ohm). Higher values are proportional to higher levels of migration ability. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$ vs. pEF. (G) Representative images of the invaded cells in chambers following incubation for 24 h and staining with calcein AM (right panel) and quantitative analysis of invasive cells (left panel). The density of green cells indicated the levels of invasion from different cell groups. (H) Representative images of adhesive cells after incubation on Matrigel for 1 h and stained with calcein AM (right) with corresponding quantitative analysis of adhesive cells (left). The density of green cells indicated the levels of adhesion from different cell groups. Data are presented as the mean \pm standard deviation. pEF, cells transfected with the empty plasmid vector; Psoexp, cells transfected with psoriasin plasmid.

the gene expression of psoriasin is low in these two GC cell lines, as indicated by RT-qPCR (Fig. 1C and D) and conventional PCR (Fig. 1E). Western blotting also demonstrated that the protein levels of psoriasin in AGS and HGC27 were low (Fig. 1F). Attempts were made to subculture other GC cell lines, such as NUGC-1, MKN-45, MKN7 and MKN74. Unfortunately, they were either floating (NUGC-1, MKN-45) or was difficult expand during the establishment of the stable cells lines (MKN7 and MKN74). Therefore, AGS and HGC-27 were to establish stable psoriasin-overexpressed cell lines

(AGS Psoexp and HGC-27 Psoexp) to determine the role of psoriasin in the tumorigenesis and progression of GC. The mRNA and protein levels of psoriasin was markedly increased in AGS Psoexp and HGC-27 Psoexp cells compared with the cells that were transfected with the vector alone (AGS pEF and HGC-27 pEF) as verified by RT-qPCR (Fig. 1C and D), PCR (Fig. 1E) and western blotting (Fig. 1F).

There was a significant increase in cell proliferation in psoriasin-overexpressed cells compared with the controls, which was assessed by crystal violet assay ($P<0.001$; Fig. 2A

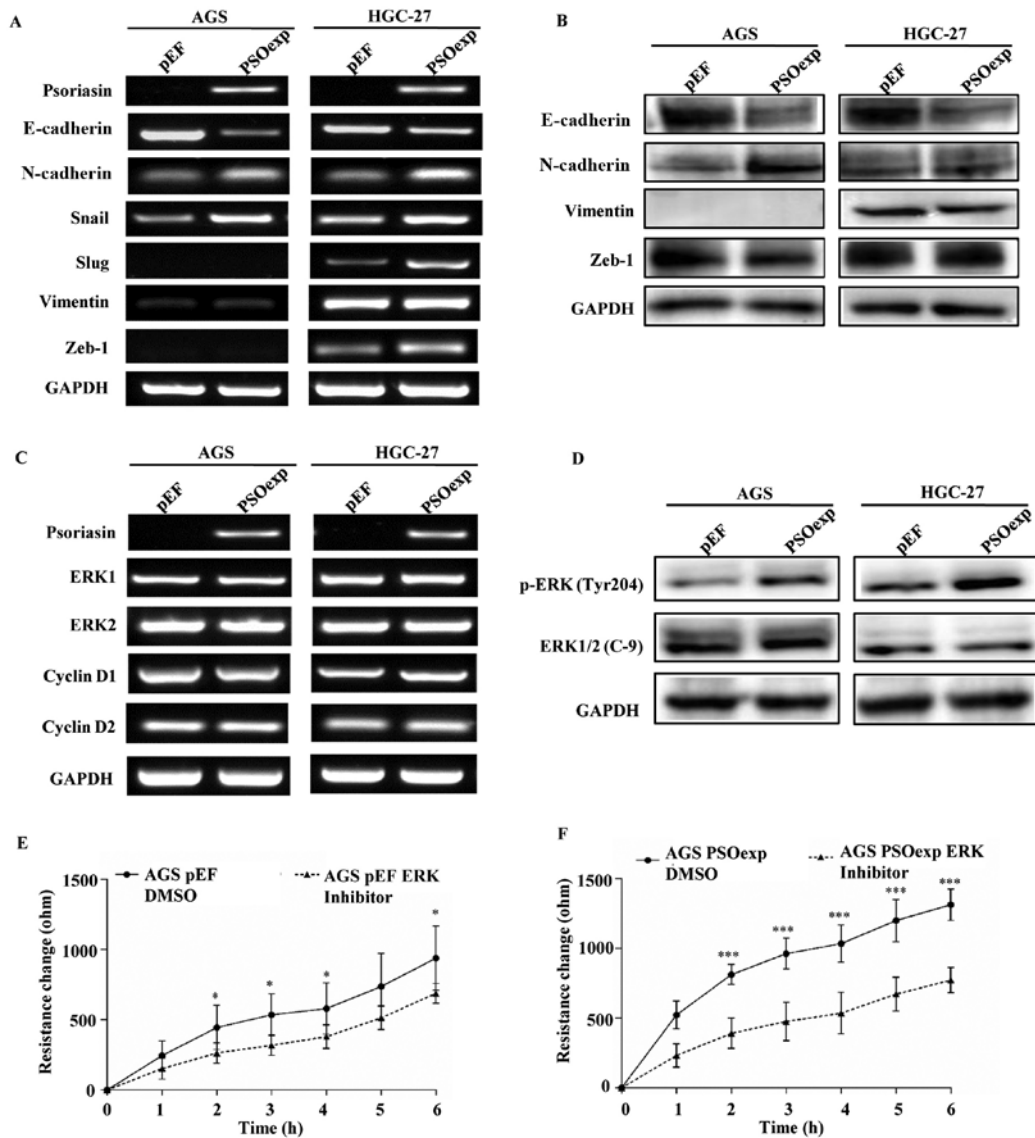


Figure 3. Effect of psoriasin overexpression on EMT and ERK expression in the gastric cancer cell lines, and effect of psoriasin on the motility and ERK expression in AGS cells that were treated with an ERK inhibitor. (A) Expression profiles of EMT in response to psoriasin overexpression in AGS and HGC-27 cells as evaluated by PCR. (B) Expression profiles of EMT in response to psoriasin overexpression in AGS and HGC-27 cells as evaluated by western blotting. (C) Evaluation of ERK activity in AGS and HGC-27 cells by PCR. (D) Evaluation of ERK activity in AGS and HGC-27 cells by western blotting. Electric cell-substrate impedance sensing analysis demonstrating migration of (E) AGS pEF and (F) AGS PSOexp cells following treatment with 1 μ M ERK inhibitor. Data are presented as the mean \pm standard deviation. EMT, epithelial-mesenchymal transition; PCR, polymerase chain reaction; pEF, cells transfected with the empty plasmid vector; PSOexp, cells transfected with psoriasin plasmid; Zeb-1, zinc finger E-box-binding homeobox 1; ERK, extracellular signal-regulated kinase; p-, phospho-; DMSO, dimethyl sulfoxide.

and B). Similarly, cell colony formation was also enhanced in the psoriasin-overexpressed cells (Fig. 2C and D). However, there was no effect on the cell cycle profile following psoriasin overexpression (data not shown).

As psoriasin expression was positively associated with lymph node involvement and distant metastasis (Table II), the migratory and invasive abilities of GC cells were evaluated *in vitro*. The Matrigel invasion assay and ECIS assay demonstrated that the overexpression of psoriasin dramatically enhanced migration (Fig. 2E and F) and invasion (Fig. 2G) in GC cell lines compared with the vector controls. Furthermore, upregulation of psoriasin significantly decreased cell-matrix adhesion in HGC-27 cells, but not in AGS cells (Fig. 2H). Together, these data suggest that psoriasin may function as an inducer of cell migration and invasion in GC.

Expression of epithelial-mesenchymal transition (EMT) markers in GC cells. Several EMT-associated molecules have been implicated in the development and progression of GC by altering the malignant properties of cancer cells (14,27). Therefore, the expression of epithelial markers, mesenchymal markers and EMT transcription factors were evaluated following overexpression of psoriasin by PCR and western blotting. The results demonstrated that the levels of N-cadherin and Snail expression were increased, while E-cadherin expression was decreased in AGS PSOexp and HGC-27 PSOexp cells compared with the empty vector controls. In addition, the upregulation of psoriasin promoted Slug and Zeb-1 expression levels in HGC-27 cells compared with the control cells, whereas the vimentin level was unchanged in both cell lines (Fig. 3A and B). Collectively, the activation of psoriasin may

Table III. Quantitative gene expression analysis of psoriasin in patients with gastric cancer receiving chemotherapy.

Variable	n	Psoriasin expression (median)	P-value
Tissue			0.005
Tumor	87	0.40	
Adjacent normal	83	1.09	
Sex			0.816
Male	63	0.41	
Female	24	0.33	
Differentiation			
Differentiated	20	0.53	
Undifferentiated	59	0.41	0.500 ^a
Others	8	0.24	0.102 ^a
Tumor size (mm)			0.202
>50	9	0.41	
<50	78	0.29	
Depth of invasion			0.116
T1-2	9	0.19	
T3-4	78	0.41	
Lymph node status			0.891
Negative	16	0.34	
Positive	71	0.41	
TNM staging			0.113
I+II	9	0.34	
III+IV	78	0.48	
Response to neoadjuvant chemotherapy			0.023
Response	30	0.24	
No response	57	0.50	

^aCompared with differentiated type; Mann-Whitney U test for comparisons between two groups; Kruskal-Wallis test for multiple group followed Tukey honest significant difference as post hoc test. TNM, tumor-node-metastasis.

alter the patterns of EMT in GC cells, promoting cell invasion and migration.

Psoriasin enhances cell migration via activating ERK signaling in GC cells. The ERK signaling cascade is one of the key signaling pathways that regulates cell proliferation, invasion and migration by controlling multiple nuclear and cytoplasmic targets in cancer (28). In the present study, the effect of psoriasin on the ERK signaling pathway was investigated in GC. The upregulation of psoriasin significantly promoted the level of phosphorylated ERK (Tyr 204) protein, without altering the total ERK1/2 mRNA and protein levels in AGS and HGC-27 cells, compared with the control cells (Fig. 3C and D).

By contrast, there was no difference in the levels of cyclin D1 and cyclin D2 mRNA between the control cells and

the cells that overexpressed psoriasin (Fig. 3C). Therefore, the effect of psoriasin on the viability of GC cells was unlikely due to the regulation of these regulatory proteins of cyclin-dependent kinases.

Treatment with an ATP-competitive ERK inhibitor suppressed the migratory ability of AGS cells (PSOexp cells and control cells), particularly in AGS PSOexp cells (Fig. 3E and F). Together, these results suggest that psoriasin may enhance cell migration at least partially via activation of ERK. Further study is required to determine whether ERK is a direct substrate of psoriasin.

Psoriasin overexpression increases resistance to cisplatin in GC cells. To determine the effect of psoriasin on cisplatin resistance in GC, the gene expression of psoriasin was analyzed in specimens from patients with GC that received pre-operative chemotherapy (n=87). The results indicated that levels of psoriasin expression were higher in patients that exhibited no response to neoadjuvant chemotherapy (P=0.023; Table III). Flow cytometry analysis of cleaved PARP was performed to evaluate the levels of apoptosis in the stable cell lines that were treated with cisplatin. As presented in Fig. 4A and B, in the presence of cisplatin, the less AGS PSOexp cells expressed cleaved PARP protein (8.95%) than the AGS pEF cells (16.87%), suggesting an increase in resistance to cisplatin. However, such increase was not observed in HGC-27 cells, as the cleaved PARP expression was not markedly altered between the HGC-27 PSOexp cell line (63.03%) and the HGC-27 pEF cell line (59.38%; Fig. 4A and B). The cell cycle analysis indicated that in response to the cisplatin treatment, a higher portion of AGS cells was arrested in the G2/M phase compared with PBS treatment in AGS pEF cells (58.68% vs. 22.51%) and AGS PSOexp cells (61.04% vs. 23.55%). However, there was no difference in cell cycle profile between the AGS PSOexp and AGS pEF cell lines in the presence of cisplatin. By contrast, the S-phase population of the HGC-27 PSOexp cells was increased compared with the pEF control cells in the presence of cisplatin (44.68% in HGC-27 pEF cells and 60.84% in HGC-27 PSOexp cells; Fig. 4C-F). Furthermore, psoriasin overexpression in AGS cells inhibited the cisplatin-induced increase in the levels of p53 and p21 proteins compared with the cells transfected with the backbone pEF plasmid (Fig. 4G and H). However, the cell viability assay showed that although psoriasin overexpression improved survival rates in the presence of particular doses of cisplatin (e.g. 6.25 μ M) in transfected AGS and HGC-27 cells compared with the pEF controls (P<0.05), the overall dose-dependent cytotoxic effect of cisplatin on the cell lines was similar to the control cell lines (data not shown). Therefore, the upregulation of psoriasin in GC cells may desensitize cells to cisplatin by promoting the proportion of cells in the S-phase or inhibiting apoptosis, instead of a direct effect via cellular cytotoxicity.

Discussion

Psoriasin is dysregulated in multiple solid tumors and performs multiple roles in a variety of pathophysiological processes, which are closely associated with tumorigenesis and cancer progression. However, knowledge on the

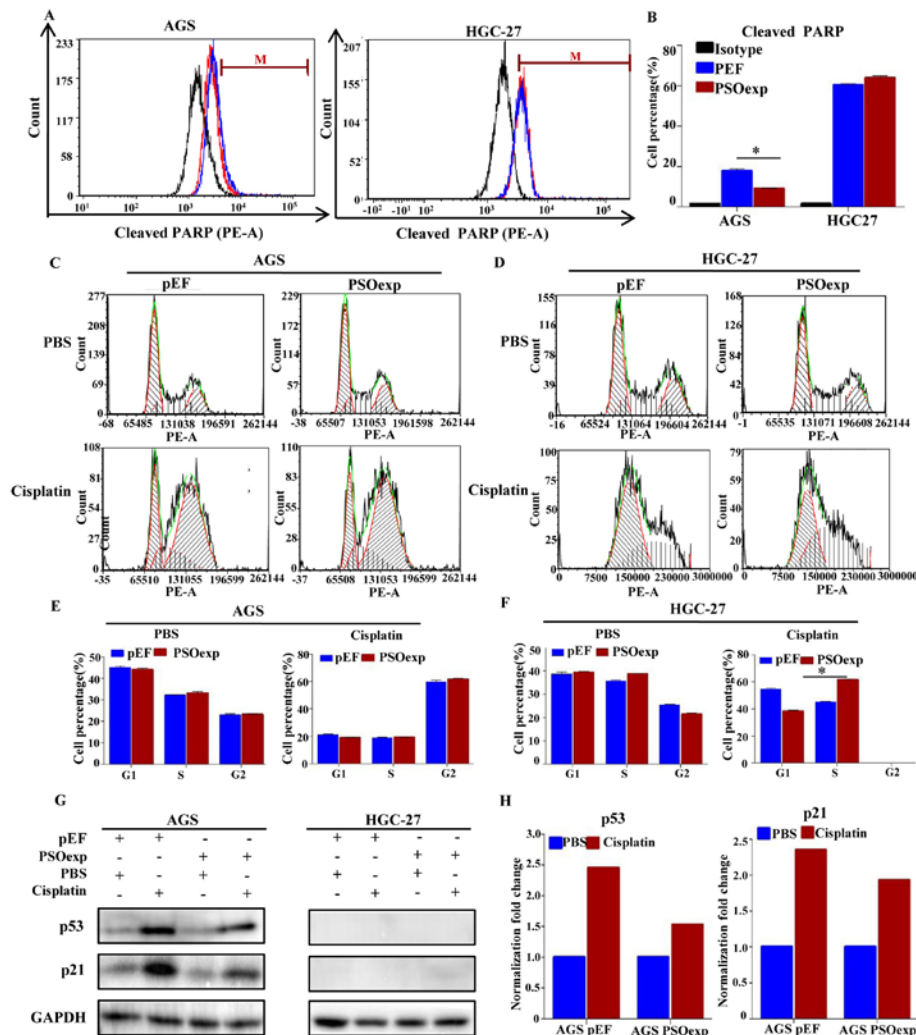


Figure 4. Effect of psoriasin overexpression on resistance to cisplatin in gastric cancer cells. (A) Flow cytometry analysis of the cleaved PARP levels in AGS and HGC-27 cells in response to treatment with 16.6 μ M cisplatin for 24 h. Black line, isotype; red line, pEF cells; blue line, PSOexp cells. (B) Cell percentage in cleaved PARP protein production in AGS and HGC27 cell lines in treatment with cisplatin by flow cytometry. (C) Changes in AGS cells (pEF and PSOexp) cycle profile following treatment with 16.6 μ M cisplatin for 24 h. (D) Changes in HGC-27 cells (pEF and PSOexp) cycle profile following treatment with 16.6 μ M cisplatin for 24 h. (E) Histogram of AGS cell cycle distribution. (F) Histogram of HGC-27 cell cycle distribution. (G) Levels of p21 and p53 proteins in AGS and HGC-27 cell lines that were treated with cisplatin as indicated by western blotting. (I) Fold change in p21 and p53 protein production in AGS cells in response to cisplatin as indicated by densitometric quantification of western blotting. The data from western blotting analysis were normalized using GAPDH. Data are presented as the mean \pm standard deviation. * $P < 0.05$ vs. relevant controls. PARP, poly(ADP-ribose) polymerase; pEF, cells transfected with the empty plasmid vector; PSOexp, cells transfected with psoriasin plasmid; PBS, phosphate-buffered saline.

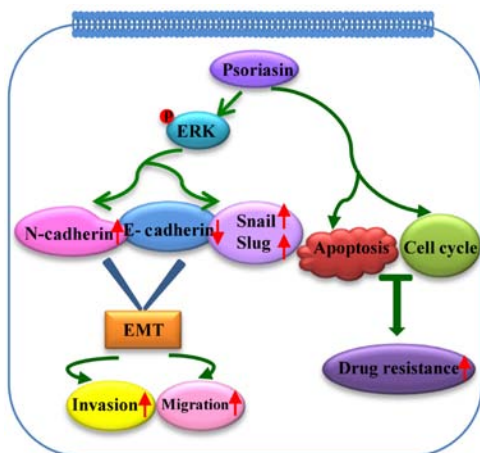


Figure 5. Schematic illustration of the potential roles of psoriasin in gastric cancer cells. p-, phospho-; ERK, extracellular signal-regulated kinase; EMT, epithelial-mesenchymal transition.

expression patterns and biological function of psoriasin in the progression of GC remains limited. In the present study, there was an increased level of psoriasin mRNA expression in GC tissues compared with adjacent normal tissues. Furthermore, this increase in psoriasin expression was also associated with lymph node involvement in patients, distant metastasis and advanced stages of the disease. These findings are consistent with the previous studies on cervical cancer and breast cancer (18,29). Additionally, Kaplan-Meier plot analysis indicated that higher psoriasin expression led to worse overall survival and disease-free survival, which was supported by the outcomes of meta-analysis from the Kaplan-Meier Plotter (gastric cancer) database (<http://kmplot.com/analysis>; data not shown). The association between psoriasin expression and clinicopathological characteristics indicates that psoriasin has a role in the development and progression of GC.

To further clarify the roles of psoriasin in gastric carcinogenesis and progression, the effects of upregulating psoriasin on cell function were evaluated. In this study, overexpression of psoriasin in AGS and HGC-27 GC cell lines promoted cell proliferation, migration and invasion, and inhibited adhesion in the HGC-27 cell line. A recent study reported that psoriasin may promote cyclin activation and cell cycle progression in pancreatic cancer cells (30). However, in the present study psoriasin had no effect on cell cycle distribution and gene expression of cell cycle regulators.

EMT is a biological process that allows polarized epithelial cells to undergo multiple changes in order to acquire a mesenchymal cell phenotype, therefore enhancing cell migration, invasion and metastasis (31). Loss of E-cadherin and increased expression of N-cadherin are hallmarks of EMT, which promote the interaction of the cell with endothelial and stromal components (27,32,33). Notably, the present data demonstrated that psoriasin drives the upregulation of N-cadherin, Snail, Slug and Zeb-1 expression and loss of E-cadherin expression in GC cells. Snail, Slug and Zeb-1, which are important transcription factors, trigger EMT by coordinating the induction of mesenchymal biomarkers and the repression of epithelial biomarkers (27). Collectively, these results suggest that psoriasin may be a potent inducer of EMT, which regulates cell invasion and migration in GC. However, the precise mechanism by which this occurs in GC requires further investigation.

ERK signaling, one of the most important intracellular pathways, has multiple functions in cancer cells, including roles in proliferation, cell cycle, EMT, migration, invasion, repression of apoptosis and induction of drug resistance (34-36). In the present study, migration was notably increased by ERK activation induced by psoriasin overexpression. Furthermore, the migration of AGS cells induced by psoriasin overexpression was inhibited by treatment with ERK inhibitor. Therefore, psoriasin may promote EMT by activating the ERK pathway, leading to enhanced migration and invasion of GC cells. However, the specific mechanism by which psoriasin affects cell migration and invasion requires further investigation.

Clinical data indicated that patients that positively responded to pre-operative chemotherapy had a significantly longer survival time than non-responders (5). Many studies have reported that the S100 proteins have the ability to regulate the sensitivity of tumor cells to chemotherapeutics (37-39). For instance, S100A11 sensitizes cells to cisplatin treatment in non-small cell lung cancer (40). However, to date, there has been no investigation into the association between psoriasin expression and chemotherapy resistance in tumors. To the best of our knowledge, the present study was the first to observe that elevated psoriasin expression in patients with GC was significantly associated with a poor pathological response to chemotherapy, suggesting that psoriasin might confer resistance in GC to chemotherapeutic drugs. *In vitro* experiments demonstrated that cells with a higher level of psoriasin expression exhibited less sensitivity to cisplatin compared with the control cells. The molecular basis of resistance to chemotherapy is generally complex, involving multiple processes, including drug metabolism and transport, apoptosis and DNA repair (41). In order to investigate the underlying mechanism, the expression cleaved PARP was used as an indicator of

apoptosis and the cell cycle following treatment of cells with cisplatin. Based on the results, psoriasin may inhibit the sensitivity of GC cells to cisplatin by ameliorating cell apoptosis or inducing the S-phase arrest, depending on the molecular phenotype, epigenetic and genetic features or p53 status of the cells.

However, there were certain limitations of the present study. As AGS and HGC-27 cells were available, they were used to explore the effect of psoriasin overexpression in GC cells. It may be ideal to find a GC cell line that overexpresses S100A7 and possesses the phenotype of GC, and explore the further biological functions via psoriasin knockdown. In addition, as AGS and HGC27 cells have low tumor formation ability in mice, this study presented the role of psoriasin in GC cells via *in vitro* experiments only. In the future, another GC cell line should be used to determine the role of psoriasin *in vivo*.

In summary, to our best knowledge, this is the first report describing the role of psoriasin in GC and chemotherapy resistance in human tumors. Psoriasin expression is frequently increased in human GC cell lines and GC tissues, and its expression is associated with metastasis and a poor survival in patients with GC. Psoriasin overexpression in GC cells increased cell proliferation, migration and invasion. Furthermore, the promoting effects of psoriasin are potentially attributed to a positive effect on EMT-associated pathways via activation of ERK signaling (Fig. 5). As psoriasin overexpression leads to a higher tolerance to cisplatin in GC cells, patient with GC with a high level of psoriasin may be less sensitive to cisplatin. Therefore, the findings suggest that psoriasin has the potential as a therapeutic target in GC and may be a useful prognostic factor to predict the effectiveness of pre-operative chemotherapy in patients with GC.

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Availability of data and materials

The analysed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

LL conducted most of the experiments in this study with some assistance from YC, LY, ZZ and WJ. JJ examined the gastric cancer tissues. WJ analysed the cohort gene expression data. YC carried out flow cytometry-based apoptosis and cell cycle analysis. LL, YC, LY and WJ provided helpful discussions and critically reviewed the manuscript. WJ and YC designed and

conceived the study. LL and YC analysed all the data and wrote the manuscript. All authors commented on and approved the manuscript.

Ethics approval and consent to participate

This study was approved by Peking University Cancer Hospital Research Ethics Committee (ethics no. 2006021) and written consent was obtained from all patients involved.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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