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PA28β Regulates Cell Invasion of Gastric Cancer via Modulating the Expression of Chloride Intracellular Channel 1

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ABSTRACT

PA28β is a subunit of proteasome activator PA28. Previous study suggests that PA28β is involved in the invasiveness and metastasis of gastric adenocarcinoma (GA), however, the mechanism is not fully understood. In the present study, we showed that invasive abilities of gastric cancer cells were enhanced when PA28β being down-regulated, and were inhibited when PA28β being overexpressed. To explore the possible mechanism of PA28β associated elevated invasiveness, the protein profiles of PA28β knock down and parental negative control gastric cancer cells were compared using proteomics approach. The results revealed that there were 43 proteins were differentially expressed, among them, chloride intracellular channel 1 (CLIC1) was significantly up-regulated and selected for further functional study. Down-regulation of CLIC1 by RNA interference was able to markedly inhibit cell invasion of PA28β knock down gastric carcinoma cells. In addition, an inverse correlation between PA28β and CLIC1 expressions was also verified in GA tissue samples, suggesting that knockdown of PA28β could enhance tumor invasion and metastasis, at least in part, through up-regulation of CLIC1. Our results provide novel insight into the mechanisms of PA28β related invasiveness and metastasis of GA, and suggest new alternative approaches for GA treatment. J. Cell. Biochem. 113: 1537–1546, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: PROTEASOME ACTIVATOR 28β; CHLORIDE INTRACELLULAR CHANNEL 1; GASTRIC CANCER; INVASION

Gastric adenocarcinoma (GA) is one of the most common and serious digestive tract cancers in China. Although an increasing number of gastric cancer patients have benefited from the development of modern tumor therapies, the prognosis of this disease is still relatively poor because the tumors are highly aggressive and highly resistant to anti-cancer drugs. The overall relative 5 years survival rate of gastric cancer has a slightly increase in the past 30 years (from 16% to 27%) but is still too low [Siegel et al., 2011]. Previous study of our lab found that human proteasome activator PA28 β subunit (PA28β) showed lower expression in GA compared to a paired normal sample [Huang et al., 2008] and the decreased expression of PA28β was correlated with advanced TNM stages (T3 and T4) compared to earlier stages (T1 and T2) in GA patients [Huang et al., 2010], implying a crucial role of PA28β in invasiveness of gastric cancer.

PA28 is a heteromeric protein complex consisting of PA28α and PA28β subunits with molecular masses of 28.6 and 27 kDa, respectively. It binds to the cylinder end of the 20S, open the gate channel to the catalytic chamber [Förster et al., 2005; Demartino and Gillette, 2007] thus activating the proteasome to generate the antigenic peptides presented by MHC class I molecules [Goldberg et al., 2002; Cerruti et al., 2007; Textoris-Taube et al.,

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2007; Yamano et al., 2008]. In PA28-deficient mice, the cytotoxic T lymphocyte (CTL) response was impaired, and the assembly of immunoproteasomes was greatly inhibited [Preckel et al., 1999]. A change in expression of PA28β is associated with several different cancers [Ebert et al., 2005; Perroud et al., 2006].

Invasiveness and metastasis are the major causes of death for cancer patients; development of the invasive phenotype of cancer cells is described as cell attachment, local proteolysis and cell migration. However, the molecular mechanism involved in these processes are complicated and far from being fully elucidated. In the present study, PA28β expression was attenuated by siRNA, and the mechanisms of PA28β in the regulation of invasiveness of gastric cancer cells was studied using a proteomic approach of 2-DE and MALDI-TOF/TOF MS.

MATERIAL AND METHODS

CELL LINES AND TISSUES

Human gastric cancer cell lines (SGC-7901, AGS-1) were obtained from the Shanghai Institute of Cell Biology at the Chinese Academy of Sciences (Shanghai, China). Human embryonic kidney cell line 293A were obtained from the American Type Culture Collection. Cells were grown at 37°C in a humidified atmosphere containing 5% CO2. The histological specimens were retrieved from the archives of Union Hospital of Fujian Medical University (Fuzhou, Fujian). The clinical pathological characteristics of these samples are shown in supplementary material (Table S1). The tissue samples were used with consent of the patients. This study was approved by the Ethical Committees of Fujian Medical University.

GENERATION OF RECOMBINANT ADENOVIRUSES

The siRNAs against PA28β were chemically synthesized (Shanghai GenePharma Co.) for targeting different regions of the gene as follows: PA28β-358 (5′-TCGAGTGATTACATGGATCCAATTTT-CAAGAGAAATTGGAT CCATGTAATCACCA-3′ and 5′-AGCTTGGT-GATTACATGGATCCAATTTCTC TTGAAAATTGGATCCATGTAAT-CAC-3′) for nt 358–376 of PA28β, and PA28β-467(5′-TCGA-GGGAAGCTTTCCAGACAATTTTCAAGAGAAATTGTCTGGAAAGC-TTCCACA-3′ and 5′-AGCTTGTGGAAGCTTTCCAGACAATTTCTCT-TGA AAATTGTCTGGAAAGCTTCCC-3′) for nt 467–485 of PA28β. In addition, siRNA-NC(5′-TCGAGCTCCGAACGTGTCACGTTTTTCAA-GAGAAAACGTGACACGTTCGGAGAAA-3′ and 5′-AGCTTTTCTC-CGAACGTGTCACGTTTTCTCTTGAAAAACGTGACACGTTCGGAGC-3′), sequences that do not target any known gene product, were used as a negative control. For the construction of the adenoviral PA28β RNA interfering (RNAi) vectors, annealed double-stranded hairpin siRNA oligonucleotides were inserted into the *Xho*I–*Hin*dIII site of the adenoviral siRNA shuttle vector pRNAT-H1.1/Adeno (GenScript, Piscataway, NJ) containing the polymerase-III H1-RNA gene promoter. The coding region of PA28β was amplified by PCR with the following primers designed according to PA28β sequences (GenBank accession number, NM_002818): forward, 5′-CTAGC-TAGCATGGCCAAGCCGTGTGG-3′, reverse, 5′-CCGCTCGAGTCAG-TACATAGATGGCTT-3′. The PCR product was digested with *Nhe*I and *Xho*I (New England Biolabs, Beverly, MA) and inserted into the *Nhe*I–*Xho*I site of the shuttle vector pShuttle-IRES-hrGRP-1 expressing green fluorescent protein (GFP). DNA sequencing and restriction enzyme digestion were used to confirm the correctness of the sequence and orientation of the inserts. Recombinant adenoviruses were prepared using the AdEasy™ XL Adenoviral vector system (Stratagene, La Jolla, CA) following the manufacturer's instructions. Briefly, *Pac*I linearized PA28β-specific siRNA and PA28β constructs or empty, control vector pShuttle-IRES-hrGRP-1 was transformed separately into competent *Escherichia coli*, BJ5183, harboring pAdEasy-1. The homologous recombined plasmids: pAd-358, pAd-467, pAd-NC, pAd-PA28β, and pAd-GFP were verified by restriction endonuclease digestion and sequencing. *Pme*I linearized plasmids were subsequently transfected into and propagated in 293A cells, and viral supernatants, that is, Ad-358, Ad-467, Ad-NC, Ad-PA28β, and Ad-GFP were harvested 14 days after transfection. After six cycles of freezing and thawing, cell debris was removed by centrifugation at 12,000*g* and the virus stock was stored at −80°C. Gastric cancer cells were infected with various adenoviruses at the same multiplicity of infection (MOI). Subsequent experiments were conducted 72 h after the initial addition of the virus. The efficiencies of the adenovirus-mediated gene transfers were between 80% and 95%.

IMMUNOBLOTTING

The cells were collected using RIPA protein lysis buffer (Pierce Company, Rockford, IL). The protein concentration was quantified with a BCA Protein Quant Kit (Bio-Rad, Hercules, CA). The protein extracts (35 μg) from cultured cells were electrophoresed using SDS–PAGE and were transferred to a Hybrid-PVDF membrane (Amersham Life Sciences, Piscataway, NJ) by an electrophoretic transfer method treated using 20% methanol in a Tris–glycine buffer (25 mM Tris–HCl, pH 8.0, 0.2 M glycine, 0.1% SDS). After blocking with TBS (150 mM NaCl, 10 mM Tris–HCl, pH 8.0) containing 5% BSA, the membrane was incubated for Western blot analysis with a rabbit anti-PA28β polyclonal antibody (1:1,000 dilution; Cell Signaling Company, Danvers, MA), a goat anti-LTA4H (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), a mouse anti-CLIC1 (1:1,000 dilution; Santa Cruz Biotechnology) or mouse anti-JAB1 (1:1,000 dilution; Santa Cruz Biotechnology) at room temperature for 2 h, followed by incubation with an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (1:5,000 dilution; Santa Cruz Biotechnology), donkey anti-goat secondary antibody (1:5,000 dilution; Santa Cruz Biotechnology) or goat anti-mouse secondary antibody (1:5,000 dilution; Santa Cruz Biotechnology). The immunoreactive protein bands were detected with CDP-Star chemiluminescent (Roche, Mannheim, Germany) and captured with X-ray film. Mouse anti-β-tubulin (1:1,000 dilution, Santa Cruz Biotechnology) was used as a loading control for Western blot analysis.

2D SAMPLE PREPARATION

After 72 h of incubation with the adenovirus, SCG7901 cells were washed three times with wash buffer (100 mM Tris–Cl pH 8.0, 1 M MgOAc), and lysed with lysis buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and 40 mM DTT, and 2% pH 3–11 NL IPG

buffer, 30 mM Tris-Base and 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 40,000*g* for 1 h, the supernatants were collected and the protein concentration was determined using a 2D Quant Kit (GE Healthcare Amersham Biosciences, Piscataway, NJ). The cell supernatants were stored at −80°C until use for electrophoresis.

2D ELECTROPHORESIS AND IMAGE ANALYSIS

A total of 600 μg protein was adjusted to a volume of 450 μl with rehydration solution containing 7.5 M urea, 4%CHAPS, 2 M thiourea, 18 mM DTT, and 0.5% pH 3–11 NL IPG buffer. The Immobiline DryStrip reswelling tray was used for rehydration of the 24 cm length IPG strip. The IPG strips were overlayed with Immobiline DryStrip Cover Fluid, and allowed to rehydrate overnight (16 h). The strips were then transferred to the strip holders, and isoelectric focusing (IEF) was performed using an Ettan IPGphor II Isoelectric Focusing system. The protocol for IEF was 200 V, 1 h; 400 V, 1 h; 2,000 V, 20 min; 4,000 V, 20 min; then continued at 8,000 V until a total of 96 kVh. The focused Immobiline DryStrip gels were equilibrated in SDS equilibration buffer and transferred onto 12% SDS–PAGE gel for second dimension electrophoresis running in 2 W each gel for 1 h and 15 W each gel until the bromophenol blue dye reached the bottom of the gel. The resulting SDS–PAGE gel was stained with Coomassie Blue R350 overnight. Triplicate gels for each sample were run to achieve reproducible 2-DE results.

After scanning and imaging, the protein spots on the gel were analyzed automatically, and the differentially expressed protein spots were detected with the Imaging Master 2D 5.0 analytical software, which was followed by a more detailed manual matching process to correct inappropriate matching pairs. The Student's *t*-test was performed on the quantitative analysis of the 2D gels; two fold or greater differentially expressed protein spots ($P < 0.05$) were excised from the SDS–PAGE gels for further identification by MALDI-TOF/TOF MS.

MALDI-TOF/TOF MS IDENTIFICATION OF THE DIFFERENTIALLY EXPRESSED PROTEIN SPOTS

The gel spots were destained, dehydrated then digested with trypsin overnight. Peptides were extracted and MALDI-TOF/TOF MS analysis was carried out. The analysis was performed using a 4700 Proteomics Analyzer (TOF/TOF TM) (Applied Biosystems, Foster City, CA) equipped with a 355-nm Nd:YAG laser. The instrument operated in the positive ion reflection mode at a 20 kV accelerating voltage using the batch mode acquisition control. Reflector spectra were obtained over a mass of 700–3,500 Da. Data from PMF and MALDI-TOF-MS/MS were analyzed using the program MASCOT (Matrix Science, London, UK) against Swiss-Prot database with GPS explorer software (Applied Biosystems). The following parameters were used in the search: human species, protein molecular mass ranged from 700 to 3,500 Da, trypsin digest with one missing cleavage, peptide tolerance of 0.2 Da, MS/MS tolerance of 0.6 Da, and possible oxidation of methionine. Tryptic autolytic fragments and contamination were removed from the data set used for the database search.

RNA INTERFERENCE AND OVEREXPRESSION OF CLIC1

Two siRNAs against CLIC1 were designed at the Whitehead Institute web-server (http://jura.wi.mit.edu/bioc/siRNAext/) and chemically synthesized (Shanghai GenePharma Co.) for targeting different regions of the gene as follows: CLIC1-133: 5′-GAGCUUGUG-UUGUGCUGAATT-3′ and 5′-UUCAGCACAACACAAGCUCTT-3′; CLIC1-146: 5′-GGACCGAGACAGUGCAGAATT-3′ and 5′-UUCUG-CACUGUCUCGGUCCTT-3′; CLIC1-1188: 5′-GGAUGAGUAGGA-CAACAUATT-3′ and 5′-UAUGUUGUCCUACUCAUCCTT-3′. In addition, a negative control, termed as siRNA-NC (5′-UUCUCC-GAACGUGUCACGUdTdT-3′ and 5′-ACGUGACACGUUCGGAGA-AdTdT-3′) were also synthesized. The above siRNAs were separately transfected into human gastric cell lines after the cells were infected with adenoviruses. Human gastric cell lines infected with adenoviruses were seeded at a density of 4×10^5 in 6-well plates. Cells grown to 40–50% confluency were transfected with synthetic siRNAs at final concentrations of 50 nM, respectively, using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions.

The full-length CLIC1 CDS was amplified by PCR from the cDNA of SGC-7901 cells. The primers contained *Bam*H I and *Not* I linkers. The primers were as follows: forward, 5′-CGCGGATCCGCTGAA-GAACAACC-3′; reverse, 5′-ATAAGAATGCGGCCGCAATTTTGA-GGGCCTTTGCC-3′. After digestion with *Bam*H I and *Not* I, the PCR product was inserted in frame into pcDNA3.1/His C plasmid expression vector (Invitrogen). Restriction enzyme digestion and DNA sequencing were used to confirm that the sequence and orientation of the construct were correct.

CELL INVASION ASSAY IN VITRO

Cell invasion assays in vitro were performed using 24-well transwells coated with Matrigel (BD Sciences). Human gastric cells were starved overnight in serum-free medium, trypsinized, and washed three times in DMEM containing 1% FBS. A total of 1×10^5 cells were suspended in 500 μl of DMEM containing 1% FBS and seeded in the upper chamber, while 750 μl of DMEM containing 10% FBS and 10 μg/ml fibronectin (BD Sciences) were added to the lower chamber. For the control, DMEM containing 1% FBS was added to the lower chamber. After incubation, Matrigel and cells remaining in the upper chamber were removed by cotton swabs. Cells on the lower surface of the membrane were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. Cells in at least six random microscopic fields (at 200×) were counted and photographed. All experiments were performed in duplicate and repeated three times.

IMMUNOHISTOCHEMISTRY (IHC)

Immunohistochemical staining for the target genes was carried out on sections of the formalin-fixed samples, including 40 cases human gastric cancer tissues. Briefly, the sections were deparaffinized with xylene for three changes and then rehydrated by transfer through graded concentrations of ethanol to distilled water, and endogenous peroxidase activity was blocked by incubation with 30 ml/L H_2O_2 in methanol for 10 min at room temperature. Then sections were submitted to antigen retrieval in a pressure cooker containing 0.01 mM sodium citrate buffer for 2 min. After washing with PBS,

slides were subsequently incubated in 100 ml/L normal goat serum for 10 min at room temperature. Sections were incubated with primary CLIC1 antibodies in PBS at 1:100 dilutions for 3 h at room temperature. The slides were incubated with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody for 10 min at room temperature. Finally, the sections were reacted with 0.02% 3,3′-diaminobenzidine and 0.005% H_2O_2 in 0.05 mM Tris–HCl buffer, and counterstaining was performed with hematoxylin. Stained slides were observed under light microscopy. Positivity was established based on the presence and intensity of brown granules; reviewed independently by two pathologists. The staining results were assessed on a four-tier scale based on Ju et al. [2008] and Ebert et al. [1994]: negative was no staining, 1+ was weak staining, 2+ was moderate staining, and 3+ was strong staining. Immunohistochemical results were graded to five different scores (0, 1, 2, 3, and 4) as follows: Score 0 was no staining or 1+ staining regardless of the percent of positive cells, Score 1 was 2+ staining in ≤30% of cells, Score 2 was 2+ staining in >30% of cells, Score 3 was 3+ staining in ≤50% of cells, and Score 4 was 3+ staining in >50% of cells.

STATISTICAL ANALYSIS

Statistical analysis was performed by using the SPSS statistical package (SPSS 17.0, SPSS, Inc., Chicago, IL). All results are expressed as mean ± standard deviation (SD). The statistical significance of differences was tested by unpaired Student's *t*-test between two groups and ANOVA between three or more groups. Correlations were made by Spearman's test. All tests are two-sided, and a *P*-value of <0.05 was considered statistically significant.

RESULTS

INFLUENCE OF PA28β ON INVASION OF GASTRIC CANCER CELLS IN VITRO

Previous work in our lab [Huang et al., 2010] demonstrated that the expression of PA28β in advanced stage (T3 + T4 group) GA was significantly lower than that in early stage (T1 + T2 group) GA, indicating that PA28β may play an important role in the invasiveness and metastasis of gastric cancer. To challenge this hypothesis, the in vitro Matrigel coated Transwell assay was used to measure the invasion ability of gastric cells at different expression levels of PA28β. First, adenovirus-mediated RNAi was used to attenuate the expression of PA28β in gastric cancer cells. Three days after incubation with the adenovirus, the expression of PA28β in SGC-7901 cells infected with Ad-358 and Ad-467 was significantly inhibited, compared to cells infected with Ad-NC (Fig. 1A). Additionally, down-regulation of PA28β significantly increased the invasion ability of SGC-7901 cells ($P < 0.01$, n = 3, Fig. 1B,C) and AGS-1 cells (Supplemental Fig. S1, A–C). In contrast,

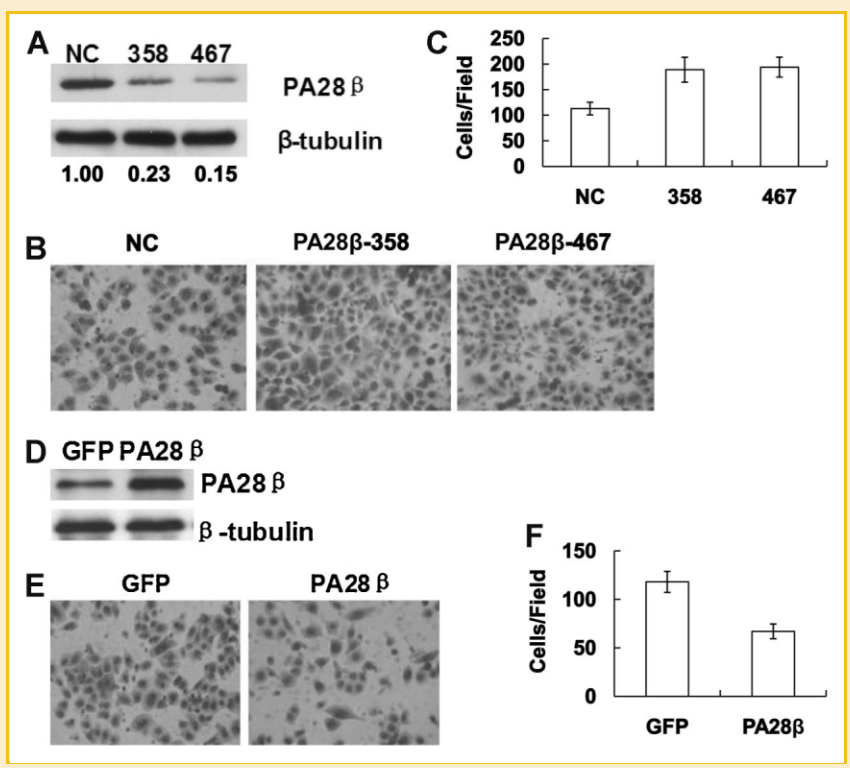

Fig. 1. The effects of PA28β expression on invasion of gastric cancer cell SGC-7901. A: Western blot analysis of PA28β expression in SGC-7901 cells with adenovirus-mediated RNA interference. Three days after infection, the expressions of PA28β in SGC-7901 cells infected with Ad-358 and Ad-467 were inhibited significantly, compared to cells infected with Ad-NC. B,C: Invasion assay. Twenty-four hours after infection with adenovirus, cells were stained with crystal violet and counted. The down-regulation of PA28β significantly increased the invasion ability of SGC-7901 cells ($P < 0.01$, n = 3). D: Western blot analysis of expression of PA28β in SGC-7901 cells infected with Ad-PA28β or empty control. E,F: Invasion assay. The overexpression of PA28β by adenovirus significantly inhibited the invasion of SGC-7901 cells ($P < 0.01$, n = 3).

overexpression of PA28β by adenovirus (Fig. 1D) significantly inhibited the invasion of SGC-7901 cells ($P < 0.01$, n = 3, Fig. 1E,F) and AGS-1 cells (Supplemental Fig. S1, D–F). These results suggest that PA28β can inhibit gastric cancer cells invasion in vitro.

PROTEOMIC COMPARISON OF PROTEIN PROFILES BETWEEN PA28β KNOCK-DOWN AND NEGATIVE CONTROL SGC-7901 CELLS

As a proteasome activator, PA28β may influence the invasion of gastric cancer cells by regulating the invasion-related protein. To address this issue, the proteomic analysis was employed to identify the differentially expressed proteins in SGC-7901 cells infected with PA28β-specific siRNA. After incubation with the adenovirus: Ad-358, Ad-467, Ad-NC, the proteins extracted from SGC-7901 cells were well-resolved on 2-DE. Upon 2-DE imaging, the proteins in SGC-7901 cells infected with different adenoviruses behaved electrophoretically in similar modes along molecular mass and pI. The electrophoretic spots were biased toward the specific pH regions on the IPG strips, whereas they were evenly distributed over the range of molecular weight from 10 to 100 kDa on 12% SDS–PAGE (Fig. 2). The images of 2-DE were further analyzed by combination

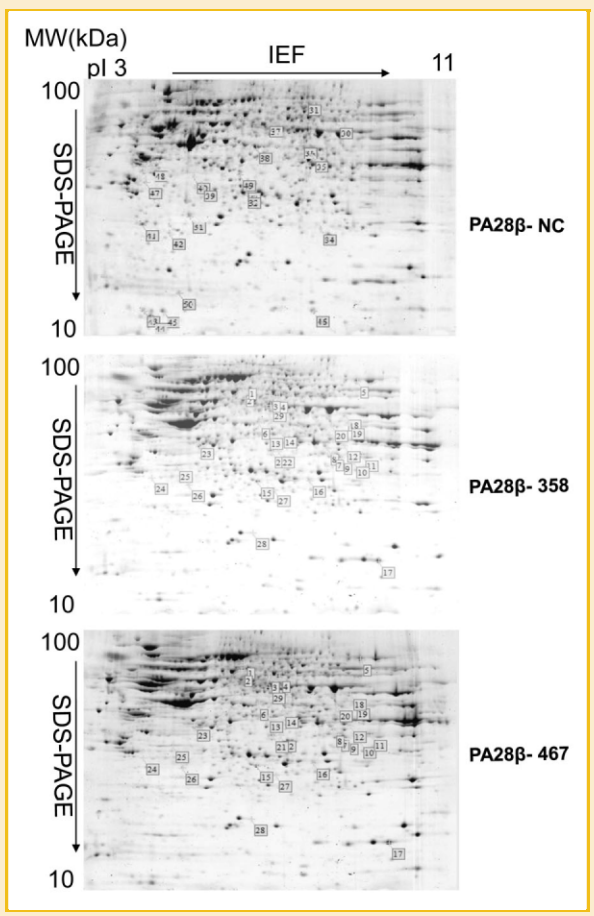

Fig. 2. Proteomic profiling of Representative 2-DE gels. 2-DE was performed with a total of 600 μg protein from SGC-7901 cells treated with PA28β-specific siRNA. The resulting SDS–PAGE gel was stained with Coomassie Blue R350 overnight. The image was analyzed with the Imaging Master 2D 5.0 analytical software.

of software, ImageMaster and manual check. The average total spots counted were 1356, 1487, and 1389 from control, Ad-358 and Ad-467-infected groups, respectively. A total of 51 spots were differentially expressed at least two fold between PA28β-specific siRNA infected SGC-7901 cells and control cells. In RNAi-infected cells, 29 proteins were overexpressed ($P < 0.05$), and 22 proteins were under-expressed ($P < 0.05$). Based on the MS data (Fig. 3), 43 spots matched with proteins involved in many biological functions, including: cell growth and differentiation, cell structure and adhesion, basic metabolism and protein degradation (Supplemental Table S2). PA28β was also identified as a protein under-expressed in RNAi-infected groups.

VALIDATION OF 2-DE PROTEOMIC RESULTS

We supposed that PA28β may inhibit cell invasion by promotion of the degradation of some oncogenes, so we focused on the invasion related genes up-regulated in PA28β knockdown cells. According to literatures, four differentially expressed proteins, including LTA4H, JAB1, CLIC1, and PA28β, were selected to verify the authenticity and reproducibility of the 2-DE proteomic results by immunoblot analysis. Beta-tubulin was used as a control to normalize the amount of protein loaded. Consistent with the results of the 2-DE proteomic analyses (Fig. 4A), LTA4H, JAB1, and CLIC1 were expressed at higher levels, while PA28β was expressed at lower levels in siRNA-infected SGC-7901 cells compared to negative control-infected cells (Fig. 4B, left panel). The expression pattern of these proteins were similar to SGC-7901 in another gastric cancer cell line, AGS-1, infected with PA28β-specific siRNA (Fig. 4B, right panel).

CLIC1 MEDIATED PA28β RELATED INVASION OF SGC-7901 CELLS IN VITRO

CLIC1 was reported as cancer metastasis-associated protein [Wang et al., 2009] so we used CLIC1-specific siRNA to explore the role of CLIC1 in the PA28β-mediated cell invasion. After infection with adenoviruses Ad-358, Ad-467, Ad-NC, CLIC1-specific siRNA were transfected into gastric cell lines, and effectively down-regulated the expression of CLIC1 (Fig. 5A). As expected, in vitro cell invasion was significantly inhibited, in accordance with cells infected with Ad-NC (Fig. 5B,C). On the other hand, overexpression of PA28β results in the reduction of the protein levels of CLIC1 (Fig. 6A) and increased invasiveness (Figs. 1 and S1), and co-overexpression of CLIC1 (Fig. 6B) can restores the levels of invasive capacity of SGC-7901 cells (Fig. 6C,D). These results suggest that, at least in part, PA28β related cell invasion is mediated by CLIC1.

INVERSE RELATIONSHIP BETWEEN THE EXPRESSION OF CLIC1 AND PA28β IN GASTRIC CANCER TISSUES

CLIC1 is up-regulated in the PA28β knock down gastric cancer cells (Fig. 4) and down-regulated in the PA28β overexpressing cells (Fig. 6A) but the transcription of CLIC1 did not changed in PA28β overexpression or knockdown cells by real-time PCR (Fig. S2), which indicated that PA28β may regulated the amount of CLIC1 by promoting its degradation. To further confirm the relationship

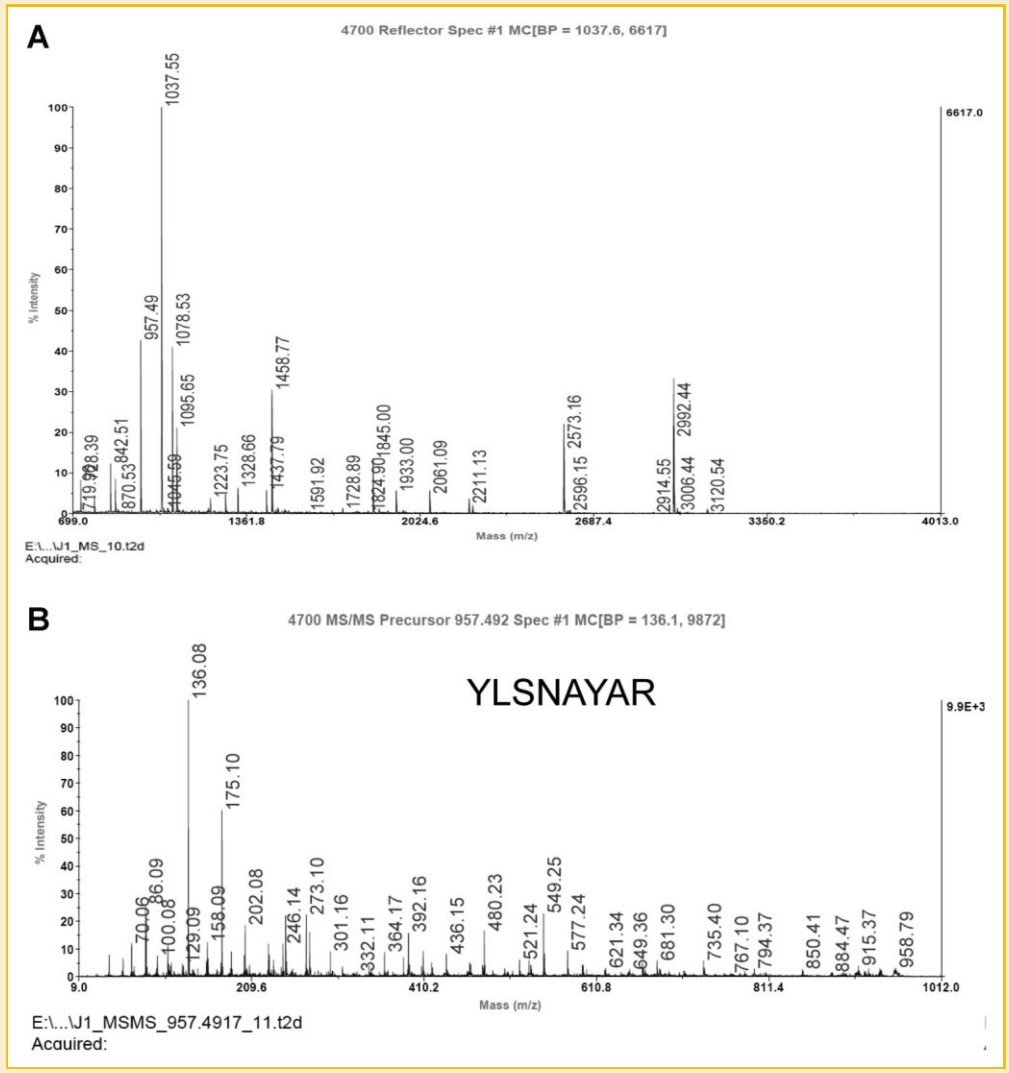

Fig. 3. MALDI-TOF-MS spectra of differentially expressed proteins. A: An example of the peptide mass map of CLIC1. B: MALDI-MS/MS sequencing result of peptide 957.49. The peptide sequence was identified as YLSNAYAR, which led to the identification of CLIC1.

between CLIC1 and PA28β levels, 40 GA tissue samples with different PA28β expression levels were examined via IHC analysis. The results showed that in the PA28β high expression GA tissues, the expression of CLIC1 were significantly lower than that with low expression of PA28β (Table I, Fisher's exact test, $P < 0.01$ and for detail see Supplemental Table S1). Figure 7 shows the expression of PA28β and CLIC1 in two representative patient samples. The correlations between CLIC1 and PA28β were investigated by Spearman's rank correlation (Supplemental Table S1). The inverse correlation between CLIC1 and PA28β was identified in GA (correlation coefficient $= -0.556$, $P < 0.01$).

DISCUSSION

In major histocompatibility complex (MHC) class I antigen processing, the proteasome plays a central role in the degradation of cytosolic proteins into appropriate peptides presented on MHC class I molecules to cytotoxic T cells [Coux et al., 1996; Koopmann et al., 1997]. PA28β was described as one subunit of PA28 that had been found to increase the peptidase activity of the proteasome [Li et al., 1999]. To date, most studies have focused on the function of PA28β involved in MHC class I antigen presentation, while only a few controversial reports show the relationship between PA28β expression and cancer [Perroud et al., 2006; Kim et al., 2008; Huang et al., 2010]. Ebert et al. [2005] identified PA28β overexpression in gastric cancer with proteomics and Perroud et al. [2006] also found that PA28β was overexpressed in renal cell carcinoma. In contrast, Kim et al. [2008] and Huang et al. [2010] found decreased expression of PA28β in lung cancer and gastric cancer. However, in these studies the change of protein level were detected by proteomics and only Huang et al. [2010] verified the change by western blot or immunohistochemistry, which improved the credibility of this study. These results should be confirmed in other cancers samples.

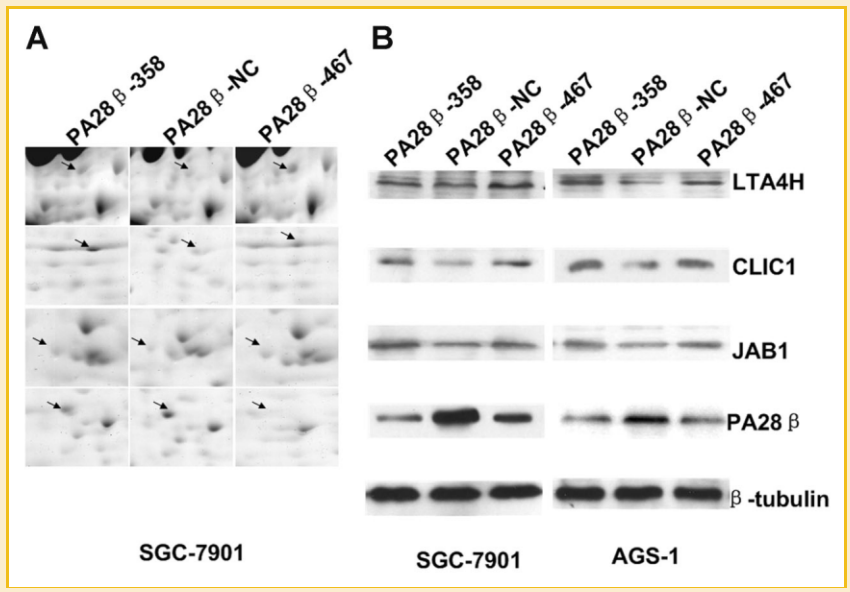

Fig. 4. Expression of LTA4H, CLIC1, JAB1, and PA28βin gastric cancer cells treated with PA28β-specific siRNA. A: 2-DE patterns of LTA4H, CLIC1, JAB1, and PA28β in SGC-7901 cells treated with PA28β-specific siRNA. B: Immunoblot analysis of protein lysates from SGC-7901 and AGS-1 cell lines treated with PA28β-specific siRNA. Lysates were separated on 12% gels, electrophoretically transferred to PVDF membranes, and the blots were probed with antibodies against LTA4H, CLIC1, JAB1, and PA28β.

Our previous study demonstrated that decreased expression of PA28β is correlated with advanced TNM stages compared to earlier stages in GA patients [Huang et al., 2010], and consistent with these findings, our present in vitro study demonstrates that gastric cancer cells with attenuated PA28β expression shows highly invasive abilities.

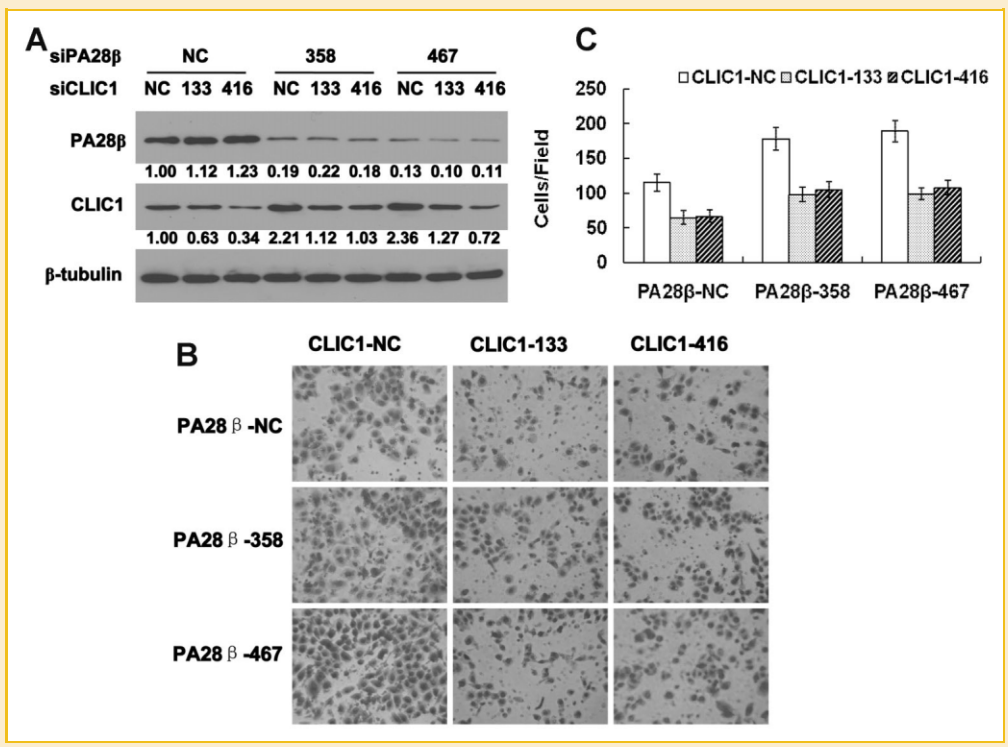

Fig. 5. Restored low invasion ability of PA28β knock down SGC7901 cells transfected with CLIC1 siRNA. A: Western blot analyze the efficiency of siRNA-CLIC1 in SGC-7901 cells. B,C: Invasion assay. After infection with adenoviruses Ad-358, Ad-467, Ad-NC, siRNA against CLIC1 were introduced into SGC-7901 cells. After 48 h, cells were stained with crystal violet and counted. The low in vitro invasion ability was restored in PA28β knock down SGC7901 cells transfected with CLIC1 siRNA. The difference was significantly ($P < 0.01$, $n = 3$).

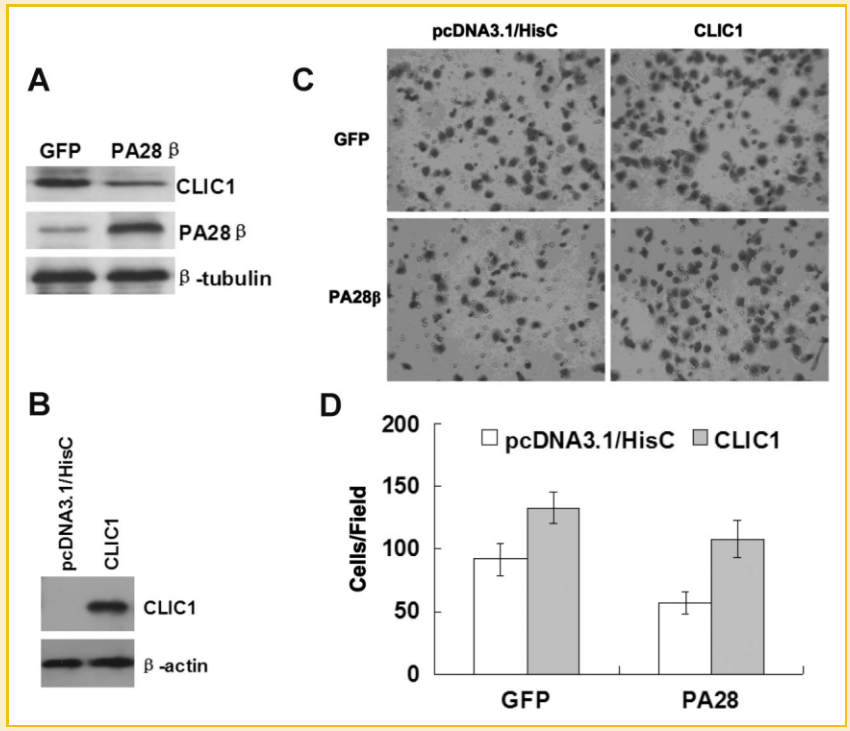

Fig. 6. Co-overexpression of CLIC1 rescued the invasiveness of PA28β overexpressed cells. A: Overexpression of PA28β decreased the protein level of CLIC1. B: The overexpression of CLIC1 was checked by Western blot using anti-X-Press antibody (Invitrogen, 1:2,000). C,D: Invasion assay. Twenty-four hour after infection with adenoviruses Ad-GFP or Ad-PA28β, SGC-7901 cells were transfected with CLIC1 overexpression plasmid or empty vector (pcDNA3.1/HisC) using Lipofectamine 2000. Forty-eight hours later, cells were stained with crystal violet and counted. The overexpression of PA28β inhibited the invasion of SGC-7901 cells, and co-overexpression of CLIC1 can restores their invasiveness ($P < 0.01$, $n = 3$).

In order to investigate the possible molecular mechanism involved in PA28β-related invasiveness and metastasis of gastric cancer, protein expression profiles of gastric cancer cells SGC-7901 infected with or without PA28β-specific siRNA were examined in this study using a proteomic approach. The results demonstrate that a total of 43 proteins are differently expressed. These identified candidate proteins can be generalized into seven groups: chaperone proteins, protein biosynthesis and regulation related proteins, transcription regulation related proteins, metabolism related enzymes, ion channel related proteins, signal transduction related proteins, and cytoskeleton proteins. Many identified proteins in this study were found to be proteasome interacting proteins (PIPs), such as proteasome subunits, chaperones, elongation factors, and ribosomal proteins. The heat shock protein's (Hsp) interaction with the proteasome is highly specific because they are induced to compensate for proteasome failure, when the proteasome activity is decreased. Hsp27 was overexpressed in gastric cancer cells treated with PA28β-specific siRNA, and was reported to assist in the unfolding of the proteins designated for degradation by the proteasome [Bercovich et al., 1997].

TABLE I. The Expression of CLIC1 in Gastric Cancer With PA28β High or Low Expression

		The expression of CLIC1 (IHC score*)			
Group	Cases	1	2	3	4
PA28β high expression	20	4	12	3	1
PA28β low expression	20	0	4	9	7

*Fisher's exact test, chi-square = 15.5, $P < 0.01$.

It is noteworthy that CLIC1 is significantly increased in gastric cancer cells infected with PA28β-specific siRNA as compared to negative control siRNA. CLIC1, also known as NCC27, is categorized as a protein belonging to the chloride ion channel (CLIC) family consisting of at least six members (CLIC1–CLIC6 proteins) [Friedli et al., 2003]. The biological functions of CLIC1 and its family include modulation of ion homeostasis, regulation of cell volume and organelles acidity, and modulation of cell division, adhesion, motility, and metastasis [Valenzuela et al., 2000; Jentsch et al., 2002; Suh et al., 2005; Wang et al., 2009]. Recently, CLIC1 protein was demonstrated to be significantly up-regulated in 67.9% gastric cancer patients, and elevated CLIC1 expression in tumor tissues was strongly correlated with lymph node metastasis, lymphatic invasion, perineural invasion, pathological staging, and poor survival in gastric cancer [Chen et al., 2007]. In addition, CLIC1 was also found to be overexpressed in hepatocellular carcinoma [Huang et al., 2004] and in colorectal cancer cells [Tomonaga et al., 2004]. All these findings indicate that overexpression of CLIC1 is closely related to tumor progression. CLIC1 was reported to be involved in several steps of angiogenesis in vitro and plays a role in mediating

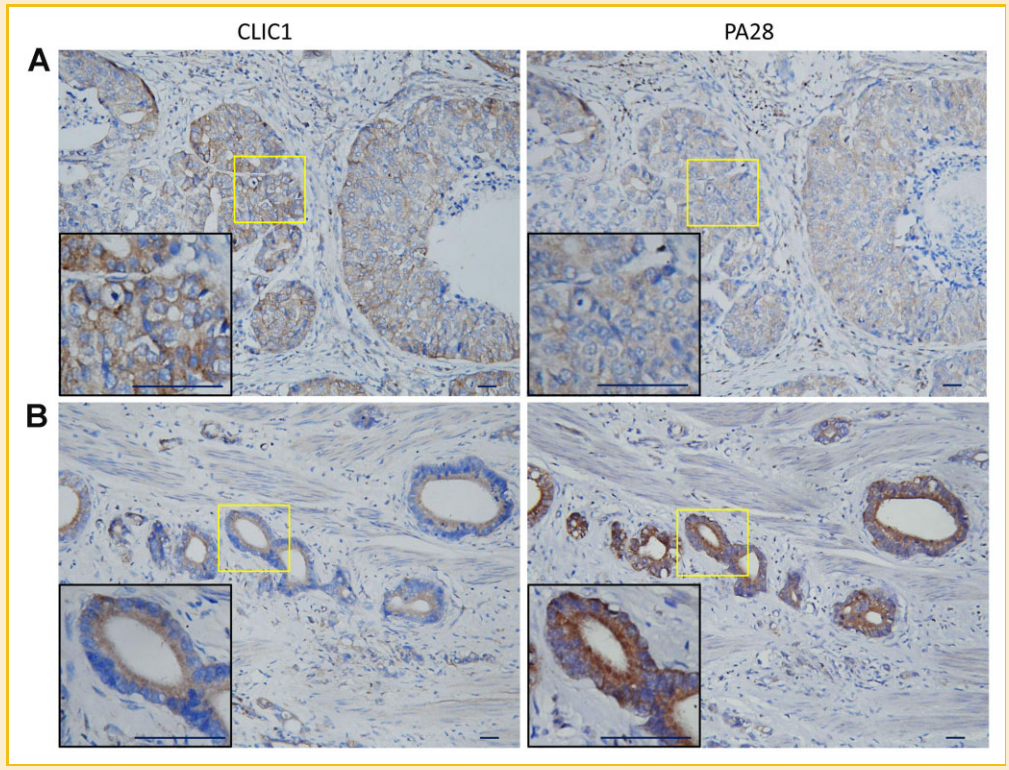

Fig. 7. The expression pattern of CLIC1 and PA28 in two representative samples from patients with gastric cancer by immunohistochemistry. A: The high expression of CLIC1 (left panel) in the PA28β (right panel) low expression sample. B: The low expression of CLIC1 (left panel) in the PA28β (right panel) high expression sample. The black bar in right bottom of each photograph represents 100 μm.

endothelial cell growth, branching morphogenesis and migration, possibly via regulation of integrin expression [Tung and Kitajewski, 2010]. CLIC1 can promote both the migration and invasion of cancer cells, and can interact directly with the actin cytoskeleton [Singh et al., 2007], we agreed with the hypothesize that CLIC1 may regulate migration of cancer cells by regulation of cytoskeletal elements [Tung and Kitajewski, 2010]. Nevertheless, the exact mechanism involved in CLIC1 associated invasion and metastasis was far from been fully elucidated and merit further study.

Molecular mechanism involving the alternation of CLIC1 is still unclear. In this study, we found that overexpression of PA28β can down-regulate and knockdown of PA28β can up-regulate the protein level of CLIC1, in addition, an inverse correlation between PA28β and CLIC1 expression was also verified in formalin-fixed GA samples by IHC. For the reason that the regulation of CLIC1 by PA28β was not at transcriptional level, as identified by real-time RT-PCR (Fig. S2), we postulate that PA28β may promote the degradation of CLIC1 at post-translational level. Combined with the findings that the invasive ability is increased in PA28β siRNA-infected gastric cancer cells, and down-regulation of CLIC1 markedly inhibits cell invasion of gastric cells treated with PA28β-specific siRNA, and vice versa, one may speculate that PA28β and CLIC1 play an important roles in GA invasiveness. We concluded that decreased expression of PA28β enhances tumor invasion and metastasis, at least in part, by up-regulation of CLIC1. Further investigations are needed to elucidate the detailed mechanisms concerning the role of PA28β in the metabolism, especially the degradation of CLIC1 during carcinogenesis of gastric cancer.

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