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# Overexpression of Myosin Phosphatase Target Subunit 1 (MYPT1) Inhibits Tumor Progression and Metastasis of Gastric Cancer

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Data Collection B  
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Data Interpretation D  
Manuscript Preparation E  
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**Background:** Myosin phosphatase target subunit 1 (MYPT1) serves as a subgroup of myosin phosphatases, and is frequently low-expressed in human cancers. However, little is known about the effects of MYPT1 in gastric cancer (GC).

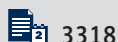
**Material/Methods:** In our study, MYPT1 expression was detected by quantitative real-time reverse transcription PCR (qRT-PCR) in GC tissues, different advanced pathological stages of GC tissues, and preoperative and postoperative patients. Kaplan-Meier analysis was used to measure the overall survival of GC patients. MYPT1 expression was analyzed by qRT-PCR and Western blot assays in GES-1 cells and GC cells. Cell proliferation, cycle, and migration and invasion abilities were detected by CCK-8, flow cytometry, and Transwell assays. E-cadherin, TIMP-2, MMP-2, MMP-9 RhoA, and p-RhoA expressions were assessed by qRT-PCR and Western blot assays in treated SNU-5 cells.

**Results:** Our results indicated that MYPT1 was down-regulated in GC tissues and cells, and is related to clinical stages and overall survival of GC. Functional research demonstrated that overexpression of MYPT1 can inhibit cell proliferation, cell cycle progression, and migration and invasion of GC cells. Many studies on mechanisms reported that overexpression of MYPT1 dramatically improved the expression levels of cell cycle-related genes (Cyclin D1 and c-myc), significantly increased epithelial marker (E-cadherin) expression, and decreased invasion-associated genes (TIMP-2 and MMP-2) expressions in SNU-5 cells. In addition, we found that MYPT1 suppressed RhoA phosphorylation.

**Conclusions:** We verified that MYPT1 inhibits GC cell proliferation and metastasis by regulating RhoA phosphorylation.

**MeSH Keywords:** **Myosin-Light-Chain Phosphatase • rhoA GTP-Binding Protein • Stomach Neoplasms • Transcellular Cell Migration**

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## Background

Gastric cancer (GC) is one of the most common malignant tumors, with the third highest mortality rate of cancer in the world [1,2]. In China, the morbidity and mortality of GC ranks high on the list of malignant tumors, and there are more than 400 000 new-onset cases each year [3,4]. At present, diagnosing and treating GC are becoming quite advanced [5–7]. However, there are no symptoms, or only mild symptoms, in the early stages of GC [8,9]. Patients lacking early diagnostic biomarkers are often diagnosed late and cannot be treated at the optimal time [9]. The main treatments for GC patients are surgical resection and chemoradiation [10,11]. However, the 5-year survival rate of GC is only 40% because of frequent recurrence [12,13]. At present, the lack of diagnostic biomarkers, prognostic indicators, and effective therapeutic targets seriously limits effective treatment of GC. Therefore, it is important for the mechanism researches of the development of GC. Recently, accumulating evidence has shown that the alteration of MYPT1 expression is involved in tumorigenesis [14–16].

The myosin phosphatase-targeting protein (MYPT) family mainly includes MYPT1, MYPT2, MYPT3, MBS85, and TIMAP, which serve as targeted and regulated subunits and can confirm the substrate-specificity of the type 1 phosphatase subunit (PP1c $\delta$ ) [17]. MYPT1, MYPT2, and MBS85 genes contain c-terminal leucine zipper domain, which can regulate the depolymerization and the protein interaction [18]. The MYPT family also can be regulated by phosphorylation activated by different protein kinases. For example, MYPT1, MYPT2, and MBS85 phosphorylated by Rho kinase finally can inhibit phosphatase activity in the process of smooth muscle contraction and Ca<sup>2+</sup> sensitivity [19].

MYPT1 is mainly involved in the RhoA/ROCK signaling pathway, and RhoA/ROCK kinase can regulate MYPT1, MYPT2, and MBS85 expression through phosphorylation, while the phosphorylation sites of MYPT1 induced by ROCK are mainly Thr696 and Thr853 [20,21]. The roles and mechanisms of MYPT1 in smooth muscle cell contraction or non-myocyte movement are: ROCK1/2 and ZIPK inhibit MYPT1 activity through promoting the phosphorylation of the T696 and T853 sites in MYPT1; CPI-17 and PH-1 can directly inhibit MYPT1, release Ca<sup>2+</sup>, activate myosin through myosin light chain kinase (MLCK), and then induce smooth muscle cell contraction or non-smooth muscle cell migration [22–24]. In addition, PKA and PKG can promote phosphorylation of Ser668, Ser692, Ser695, and Ser852 sites in MYPT1 to activate its activity. Telokin can directly activate MYPT1, MYPT1 activation can inhibit myosin light streptokinase, and then relax smooth muscle cells [25,26].

The MYPT family play important roles in the development of diseases such as cancer, hypertension, and Parkinson's disease [27–29]. Studies have indicated that MYPT1 plays vital roles

in the development and progression of cancers, such as cell cycle, migration, and invasion [16,30–33]. The compounds of MYPT1 and protein phosphatase 1 (PP1) can dephosphorylate the receptor interacting protein, and inhibit the activation of proteins [34,35]. MYPT1 can reduce the vasodilatation reaction mediated by nitrogen oxide through changing the generation of MYPT1 LZ+ [36]. MYPT1 has many different binding sites and subcellular interactions, and resistance or activation of MYPT1 expression may be a therapeutic target for tumors [37]. In this study, we demonstrated the expression level of MYPT1 in GC tissues and cells, and the relationship with clinical stages and overall survival of GC. We also verified the roles of MYPT1 in GC cell proliferation, cell cycle progression, migration, and invasion. Furthermore, we proved the mechanisms of MYPT1 in GC, such as the regulation of MYPT1 on cell cycle-related genes (Cyclin D1 and c-myc) expressions, the regulation of MYPT1 on invasion-associated genes (E-cadherin, TIMP-2, MMP-2, and MMP-9), and the regulation of MYPT1 on RhoA phosphorylation. We found that MYPT1 may be a novel therapeutic target for the treatment of GC.

## Material and Methods

### Clinical samples

Gastric cancer tissue samples and adjacent normal tissues (5 cm away from the tumor) from 68 GC patients were collected from Tongde Hospital of Zhejiang Province from February 2011 to June 2016. Informed consent was obtained from all GC patients, and ethics approval was granted by the Ethics Committee of Tongde Hospital of Zhejiang Province. All tissues were immediately put in liquid nitrogen after removal from GC patients, and stored at –80°C until used.

Peripheral blood from 43 GC patients was extracted before and 14 d after surgery. We collected 5 ml of blood and allowed it to clot at room temperature for 30 min to 2 h. Serum was isolated from all blood samples using a two-step centrifugation protocol (2000 g for 10 min at 4°C, 12 000 g for 10 min at 4°C). After separation, serum samples were added into RNase DNase-free tubes and stored at –80°C.

### Cell culture

Gastric epithelial cell (GES-1) and GC cell lines (SNU-5, MKN-45, BGC-823, SGC-7901, AGS, and HGC-27) were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). All cells were inoculated into a sterile culture bottle, and incubated in RPMI-1640 medium (HyClone, Cat. No. SH30027) with 10% fetal bovine serum (FBS, Cat. 10082-147, Gibco/Life Technologies, Norwalk, CT), 100 U/mL penicillin and 100 U/mL streptomycin

(Invitrogen, Carlsbad, CA, Cat. No. 15140-12) at 37°C and 5% CO<sub>2</sub> saturation. The culture medium was replaced every 2–3 d. Cells were digested with 0.25% trypsin ion when cell fusion reached to 80%–90%, and were inoculated to culture bottles (25 cm<sup>2</sup>) with 5×10<sup>5</sup> cells.

### Plasmid construct and transfection

To construct the pcDNA3.1-MYPT1 expression vector, the complete sequence of human MYPT1 gene was synthesized and inserted into a pcDNA3.1 (+) vector (GenePharma, Shanghai, China). SNU-5 cells (5×10<sup>5</sup> cells) at the logarithmic phase were inoculated in 6-well plates and incubated for 12 h. Then, cells were transfected with pcDNA3.1 and pcDNA3.1-MYPT1 plasmid using Lipofectamine 2000 reagent (Invitrogen, cat. no. 11668-019) and Opti-MEM reduced serum medium (Life Technologies), according to the manufacturer's instructions, when the cell fusion rate reached to 80%.

### RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the treated SNU-5 cells by using TRIzol reagent (Invitrogen).

The RNA concentration was quantitated by using the OD value. Then total RNA (1 µg) was used to synthesize complementary DNA (cDNA) using the high-capacity cDNA reverse transcription Kit (cat. no. 4368814) in a total volume of 20 µl according to manufacturer's instructions. RT-qPCR reactions were performed using SYBR Green (Applied Biosystems, cat #4368577) in a total volume of 20 µl according to the manufacturer's instructions. The reaction process was as follows: 95°C for 10 min, 95°C for 15 s for 40 cycles, and 60°C for 60 s. The results were measured on a ABI 7500 real-time PCR instrument (Applied Biosystems, Carlsbad, CA). The relative mRNA expression levels were analyzed using 2<sup>-ΔΔCt</sup> method [38]. The primers were as follows:

MYPT1: 5'-GAGCTCCGGTGGTGAAG-3' (forward),  
5'-GGCAGTGAGTCCGTCCAC-3' (reverse);  
Cyclin D1: 5'-CTTCTGTCTACTACCGCC-3' (forward),  
5'-CTCCTCTCTCTCTCTCT-3' (reverse);  
c-myc: 5'-AGCATACCTCTGTCGTC-3' (forward),  
5'-CAAGAGTTCGCTAGCTGTCA-3' (reverse);  
E-cadherin: 5'-ACT GAT TTT CCC ACG GAC CT-3' (forward),  
5'-CTC CTC GCT TTC CAT GTG TG-3' (reverse);  
TIMP-2: 5'-TTCAAAGGGCTGAGAAGGA-3' (forward),  
5'-TCAGGCTCTTCTCTGGGTG-3' (reverse);  
MMP-2: 5'-GATACCCCTTTGACGGTAAGGA-3' (forward),  
5'-CCTTCTCCCAAGTCCATAGC-3' (reverse);  
MMP-9: 5'-TTCAAGGAGACGCCATTTC-3' (forward),  
5'-AAACCGAGTTGGAACCACGA-3' (reverse);  
GAPDH: 5'-GCC ATC ACA GCA ACA CAG AA-3' (forward),  
5'-GCC ATA CCA GTA AGC TTG CC-3' (reverse).

### Western blot assay

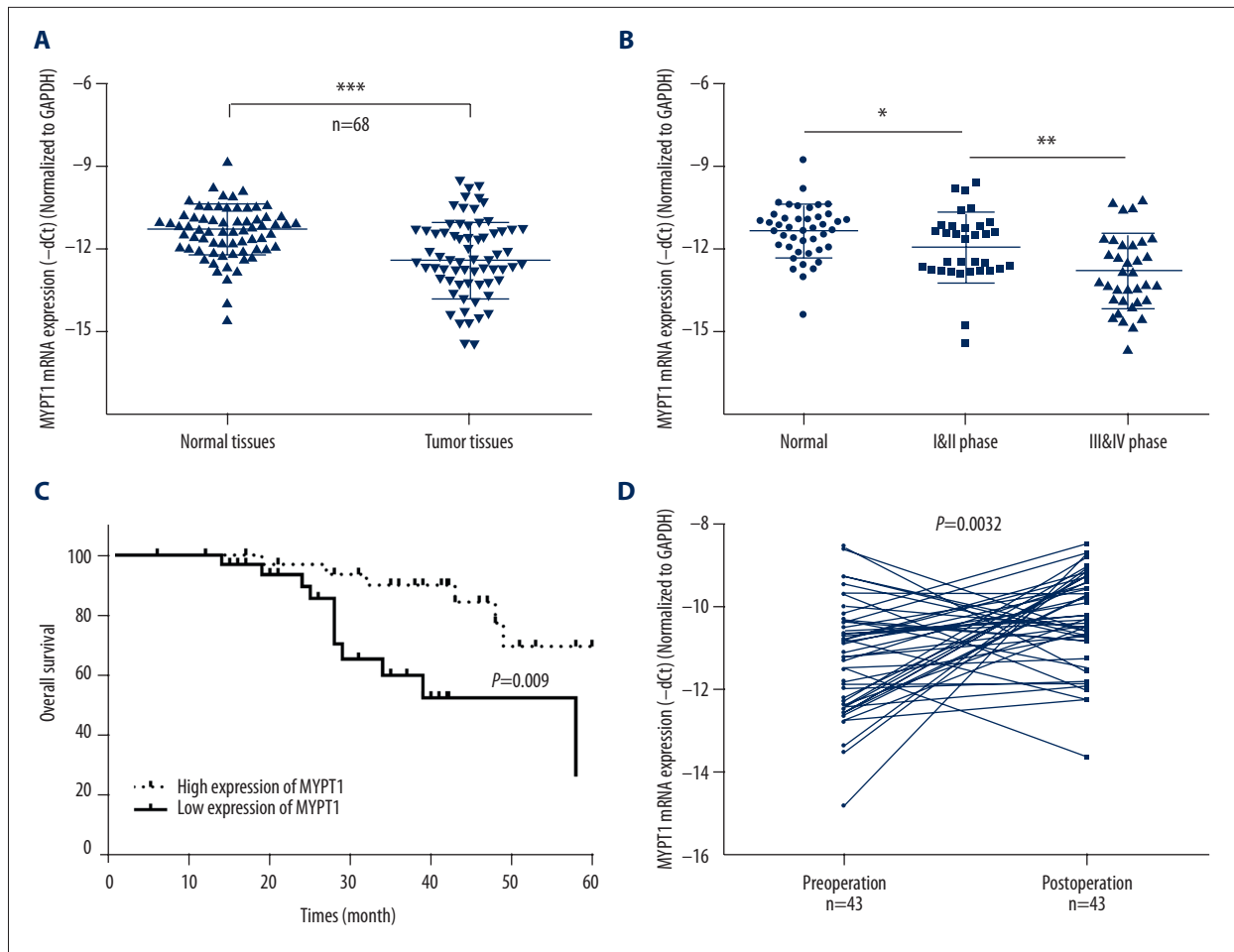
Total protein was extracted from the treated SNU-5 cells using RIPA lysis buffer (100–200 µL lysate, cat# 89901; Thermo Scientific, Rockford, IL, USA). Protein was quantified using a BCA protein quantification kit (23225, Pierce, Rockford, IL, USA). Then, 30 µg protein was separated using 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 150V, 90 min), and transferred onto a polyvinylidene difluoride (PVDF, Millipore, Billerica, MA) membrane (100 V, 90 min). The PVDF membrane was blocked with 5% non-fat dried milk in TBST solution for 1 h at room temperature, and incubated with primary antibodies at 4°C overnight. The next day, the PVDF membrane was washed 3 times with TBST solution (10 min/time), and incubated with secondary antibody (cat. number LK2003L, Sungene Biotech Co., Ltd, China) for 1 h at room temperature. The protein was detected using an enhanced chemiluminescence (ECL) substrate kit (Thermo scientific Pierce) and ImageJ software (NIH, Bethesda, Maryland, USA). The gray values of objective proteins and internal reference were analyzed by Quantity One V4.6.2 software (Bio-Rad, USA). The primary antibodies were anti-GAPDH antibody (Dilution 1: 2000; Abcam, ab8245), anti-Bax antibody (Dilution 1: 1000; Abcam, ab32503), anti-MYPT1 antibody (Dilution 1: 1000; BD Biosciences, Cat 612164), anti-Cyclin D1 antibody (Dilution 1: 1000; Cell Signaling Technology, cat. 2922), anti-c-myc antibody (Dilution 1: 1500; Abcam, ab32072), anti-E-cadherin antibody (Dilution 1: 1000; Cell Signaling Technology, Cat# 31955), anti-TIMP-2 antibody (Dilution 1: 1000; Abcam, cat. no. ab180630), anti-MMP-2 antibody (Dilution 1: 1000; Cat. No. PF023, Millipore, Darmstadt, Germany), anti-MMP-9 antibody (Dilution 1: 50; Santa Cruz, cat # sc-21733), anti-p-RhoA antibody (Dilution 1: 1000; Abcam, ab41435), anti-RhoA antibody (Dilution 1: 2000; Abcam, cat. no. ab187027).

### Proliferation assay

SNU-5 cells (5×10<sup>4</sup> cells/mL) in the logarithmic phase were seeded into 96-well plates in 200 µL of medium and transfected with pcDNA3.1 and pcDNA3.1-MYPT1 for 48 h at 37°C with 5% CO<sub>2</sub>. To detect cell proliferation, 10 µL of Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was added to each well. After 3 h, the absorbance was detected at 450 nm using a Thermo Multiskan Ex plate reader (Thermo Fisher, United Kingdom).

### Flow cytometric analysis

The SNU-5 cells (6×10<sup>7</sup> cells) at the logarithmic phase were seeded into 6-well plates and were transfected with pcDNA3.1 and pcDNA3.1-MYPT1 for 48 h in an incubator (37°C, 5% CO<sub>2</sub>). Cells were collected and treated with pre-cooled 70% ethanol (1 ml), and then the treated cells were treated with 500 µL



**Figure 1.** Relative MYPT1 expression in GC and the relationship with overall survival of GC. **(A)** The mRNA expression level of MYPT1 was detected by qRT-PCR assay in GC tissues and corresponding non-tumor tissues (n=68, \*\*\*  $P < 0.001$ ). **(B)** MYPT1 expression was measured by qRT-PCR assay in different advanced pathological stages normal (N0, n=43), I & II phase (N1 and N2, n=33), III and IV phase (N3 and N4, n=35), \*  $P < 0.05$ , \*\*  $P < 0.01$ . **(C)** Based on MYPT1 expression in GC tissues, the overall survival of GC patients was calculated by Kaplan-Meier analysis ( $P = 0.009$ ). **(D)** MYPT1 expression was analyzed by qRT-PCR assay in preoperative and postoperative patient serum (n=43,  $P = 0.0032$ ).

phosphate-buffered saline (PBS, cat#AM9625) containing propidium iodide (PI, Cat. P4170, Sigma-Aldrich, USA, 50  $\mu\text{g}/\text{mL}$ ), RNase A (100  $\mu\text{g}/\text{mL}$ , Cat# 109169), and 0.2% Triton X-100 (Sigma-Aldrich, cat. no. T8787) in the dark for 30 min. Finally, the results were obtained by using a flow cytometer, and cell cycle analysis was performed by FlowJo software (Tree Star, Ashland, OR).

### Transwell assay

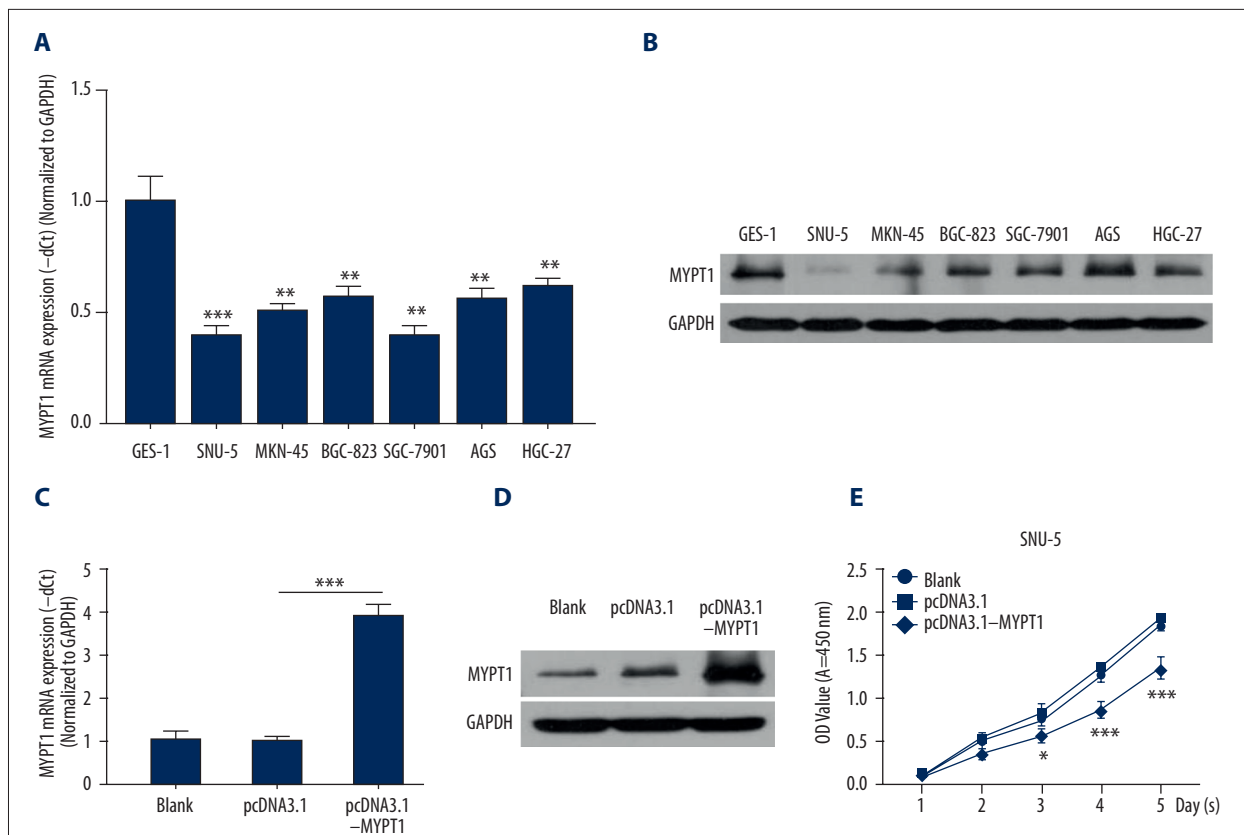
Cell migration and invasion were confirmed by detecting the ability of cells to move to another place. The cells at the logarithmic phase were digested, washed with PBS twice, and suspended in RPMI-1640 medium without FBS, and the concentration was adjusted to  $2 \times 10^5$  cells/ml. Cells suspensions (100  $\mu\text{L}$ ) were added to the polycarbonate membrane of the

upper chamber with (for the invasion assay) or without (for the migration assay) Matrigel (BD Bioscience, San Diego, CA).

The bottom chamber was filled with complete medium (500  $\mu\text{L}$ ). The cells were incubated at 37°C for 24 h. Cells on the bottom of the coated Transwell chamber were washed twice, fixed with 4% paraformaldehyde (Cat#P6148) for 30 min, and stained with 0.1% crystal violet (no. C3886-100G0; Sigma-Aldrich, St. Louis, MO) for 15 min at room temperature. After drying, the number of migrated or invaded cells was analyzed from 5 randomly selected fields under a microscope at a magnification of  $\times 100$ .

### Statistical analysis

All the experimental results are presented as mean  $\pm$  SD of at least 3 independent experiments. The data were analyzed by



**Figure 2.** Overexpression of MYPT1 inhibits GC cell proliferation. MYPT1 expression was analyzed by qRT-PCR (A) and Western blot (B) assays in gastric epithelial cell (GES-1) and GC cell lines (SNU-5, MKN-45, BGC-823, SGC-7901, AGS, and HGC-27), \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  vs. GES-1 group. (C) SNU-5 cells were treated with PBS (Blank), pcDNA3.1, pcDNA3.1-MYPT1 for 48 h, respectively. MYPT1 mRNA expression level was evaluated by qRT-PCR assay (\*\*\*)  $P < 0.001$ . (D) MYPT1 protein expression level was measured by Western blot assay in treated SNU-5 cells. (E) CCK-8 assay was performed to detect SNU-5 cell proliferation (\*  $P < 0.05$ , \*\*\*  $P < 0.001$ ).

using IBM SPSS Version 20 with the  $t$  test. Statistical significance was defined as  $P < 0.05$ .

## Results

### MYPT1 was down-regulated in GC

To explore MYPT1 expression levels in GC, we used qRT-PCR assay to measure its expression in 68 pairs of GC (tumor tissues) and adjacent non-cancerous tissues (normal tissues). The results indicated that the mRNA expression level of MYPT1 was significantly decreased in GC tissues compared with normal tissues ( $P < 0.001$ , Figure 1A). Furthermore, we found that MYPT1 expression was significantly decreased in phase I and II (N1 and N2,  $n = 33$ ) compared with normal tissues (N0,  $n = 43$ ) ( $P < 0.05$ ); and was significantly down-regulated in phase III and IV (N3 and N4,  $n = 35$ ) compared with phase I and II (N1 and N2,  $n = 33$ ) ( $P < 0.01$ , Figure 1B).

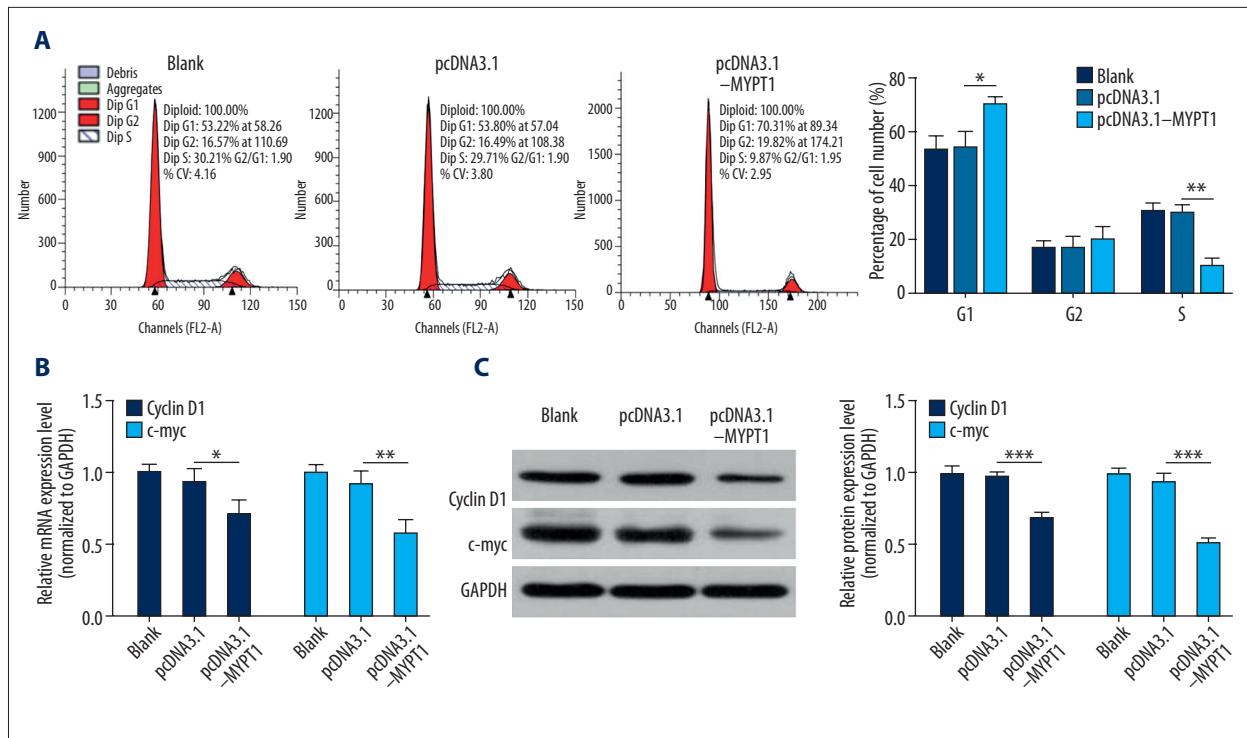
### MYPT1 expression was related to overall survival of GC

To further explore the correlation between MYPT1 expression and GC survival, Kaplan-Meier analysis was used to assess the overall survival of GC patients according to MYPT1 expression. The results revealed that GC patients with high MYPT1 expression had a dramatically longer survival time compared with those with low MYPT1 expression ( $P = 0.009$ , Figure 1C). In addition, we found that MYPT1 expression was higher in post-operative patients than in preoperative patients ( $P = 0.0032$ , Figure 1D). Therefore, MYPT1 expression was associated with survival and prognosis.

### Overexpression of MYPT1 inhibits GC cell proliferation

According to the above observations, we then demonstrated the function and mechanism of MYPT1 in GC. Firstly, MYPT1 expression was detected in gastric epithelial cell (GES-1) and GC cell lines (SNU-5, MKN-45, BGC-823, SGC-7901, AGS, and HGC-27). The results revealed that MYPT1 expression was





**Figure 3.** Overexpression of MYPT1 induces SNU-5 cell cycle arrest in G1 phase. (A) The cell cycle was analyzed flow cytometry in treated SNU-5 cells, and the values of G1, S, and G2 were shown in the bar graphs (\*  $P < 0.05$ , \*\*  $P < 0.01$ ). (B) qRT-PCR and (C) Western blot assays were performed to analyze the mRNA and protein expression levels of Cyclin D1 and c-myc in treated SNU-5 cells (\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ ).

markedly inhibited in 6 GC cell lines compared with the GES-1 cells ( $P < 0.01$ ,  $P < 0.001$ , Figure 2A, 2B). We also found that MYPT1 expression was lower in SNU-5 cells than in other GC cell lines, and we chose to use SNU-5 cells in subsequent experiments. SNU-5 cells were treated with PBS (Blank), pcDNA3.1, and pcDNA3.1-MYPT1 for 48 h, respectively. MYPT1 expression was analyzed in treated SNU-5 cells, and the results showed that MYPT1 was highly expressed in the pcDNA3.1-MYPT1 group compared with the pcDNA3.1 group ( $P < 0.001$ , Figure 2C, 2D). In addition, we proved that overexpression of MYPT1 significantly inhibited the proliferation ability of SNU-5 cells ( $P < 0.05$ ,  $P < 0.001$ , Figure 2E).

### Overexpression of MYPT1 induces SNU-5 cell cycle arrest in G1 phase

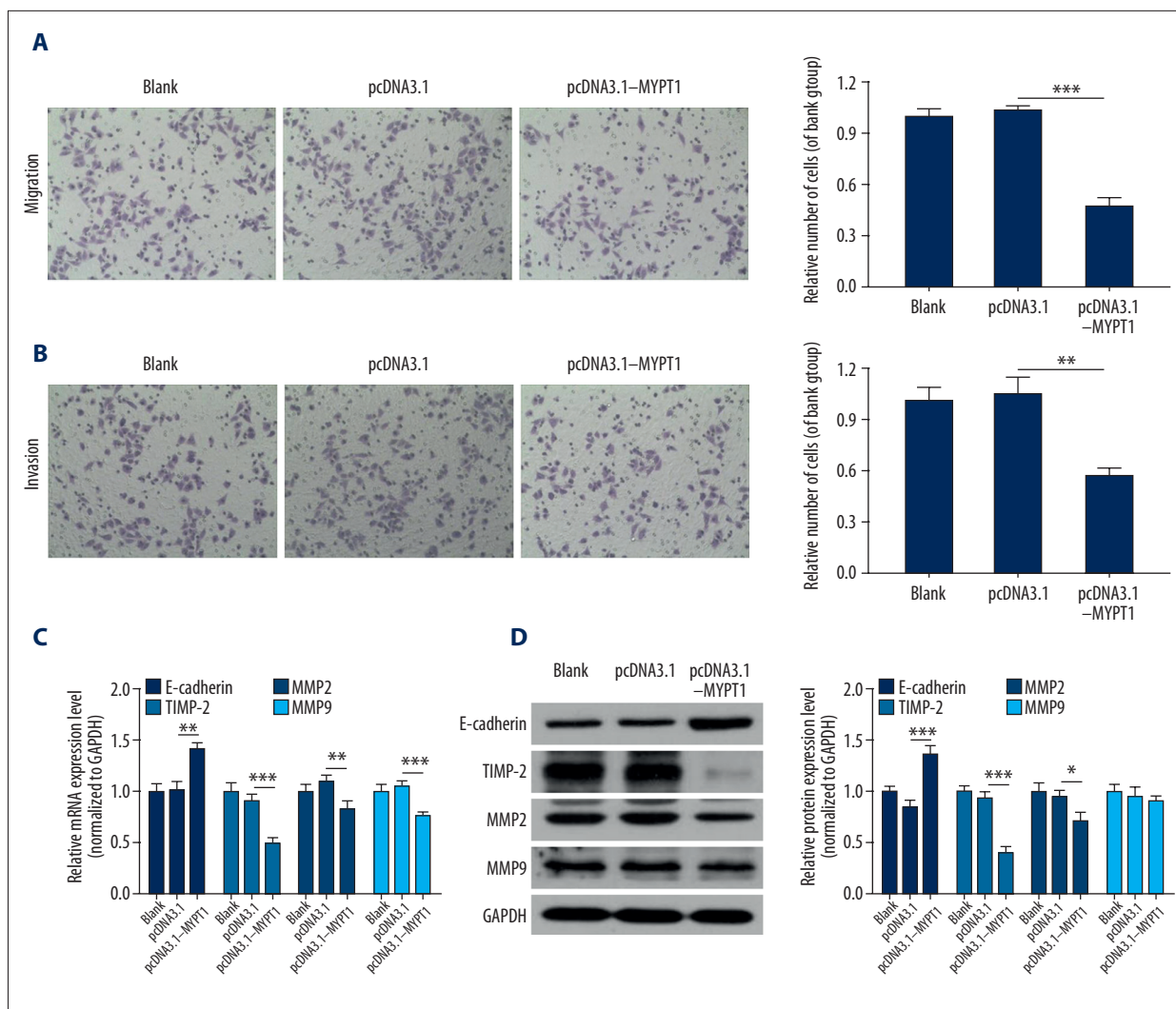
Cell cycle is connected with cell proliferation. Therefore, we further assessed the cell cycle distributions using flow cytometry. SNU-5 cells transfected with pcDNA3.1-MYPT1 showed significant G1 arrest and S phase reduction compared with the pcDNA3.1 group ( $P < 0.05$ ,  $P < 0.01$ , Figure 3A). In addition, we analyzed cell cycle-related genes (Cyclin D1 and c-myc) expressions using qRT-PCR and Western blot assays, and found that overexpression of MYPT1 dramatically improved the expression levels of Cyclin D1 and c-myc in SNU-5 cells ( $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.001$ , Figure 3B, 3C).

### Overexpression of MYPT1 inhibits SNU-5 cell migration and invasion

Because we found that overexpression of MYPT1 inhibited GC cell proliferation and induced GC cell cycle arrest, we further assessed the effect of MYPT1 on migration and invasion, and the data showed that overexpression of MYPT1 markedly inhibited the migration and invasion capacities of SNU-5 cells ( $P < 0.01$ ,  $P < 0.001$ , Figure 4A, 4B). We also analyzed the influences of MYPT1 on metastasis-associated genes (E-cadherin, TIMP-2, MMP-2, and MMP-9) expressions. As shown in Figure 4C and 4D, overexpression of MYPT1 remarkably increased E-cadherin expression and decreased TIMP-2 and MMP-2 expressions ( $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.001$ ).

### Overexpression of MYPT1 suppresses RhoA phosphorylation

Studied have shown that small GTPases, which are oncogenic genes, have important effects in the tumorigenic process [39,40]. RhoA was a major member of the Rho family of small GTPases-Ras-like proteins, which is involved in proliferation, differentiation, migration, and invasion of cancers [41-45]. Therefore, we analyzed the effect of MYPT1 on RhoA expression, and found that p-RhoA expression was obviously



**Figure 4.** Overexpression of MYPT1 inhibits SNU-5 cell migration and invasion. The migration (A) and invasion (B) abilities of the treated SNU-5 cells were measured by Transwell assays (\*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ ). (C) qRT-PCR and (D) Western blot assays were performed to detect the mRNA and protein expression levels of E-cadherin, TIMP-2, MMP-2 and MMP-9 in treated SNU-5 cells (\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ ).

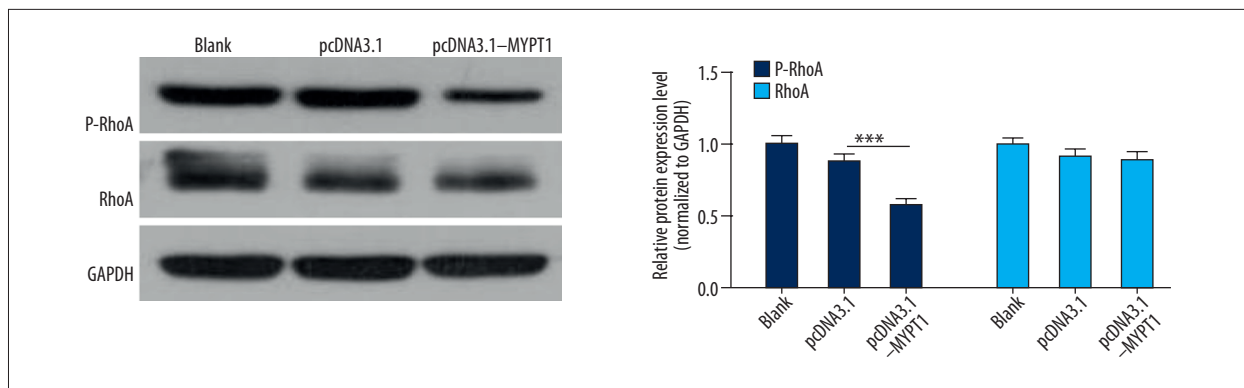
down-regulated in the pcDNA3.1-MYPT1 group compared with the pcDNA3.1 group ( $P < 0.001$ , Figure 5).

### Discussion

GC is one of the most frequently diagnosed cancers and is the second leading cause of cancer-related death worldwide [46,47]. China has the highest incidence of GC and the highest rates of GC mortality [48]. Although several strategies have been proposed for GC screening, most patients are diagnosed at advanced stage, with dismal outcome [49]. Although several molecularly targeted drugs have been developed, most advanced GC has a poor prognosis [50], and a new appropriate site for targeted therapy needs to be found. At present, MYPT1 has

been established as having a key role in regulating various biological and pathological processes in a great number of human diseases [14,51].

ROCK is a serine/threonine kinase that can participate in the regulation of cell adhesion, movement, proliferation, differentiation, and apoptosis. Myoglobin phosphatase (MLCP) and myosin light chain (MLC) are the 2 main substrates of ROCK [52]. MLCP is composed of catalytic domain, MLC-binding subunit, and non-catalytic subunits, and the action sites are located in the MYPT-1 binding subunit of MLCP [53]. Studies have indicated that myosin phosphatase-RhoA interacting protein (M-RIP) can combine Rho A/ROCK and MLCP. MYPT-1 is phosphorylated by ROCK, which causes MLCP inactivation; however, MYPT-1 phosphorylation can lead to MYPT-1 dissociation,



**Figure 5.** Overexpression of MYPT1 suppresses RhoA phosphorylation. RhoA and p-RhoA expressions were detected by Western blot assay in treated SNU-5 cells, and the relative expression levels were counted from 3 independent experiments (\*\*\*)  $P < 0.001$ .

which can block the dephosphorylation effect of MLCP on MLC. Both of them can increase the content of phosphorylated MLC, and finally result in enhanced contraction of smooth muscle [54]. In addition, ROCK can phosphorylate MLCP and directly phosphorylate MLC to increase the content of phosphorylated MLC [55].

In our study, we found that MYPT1 was significantly decreased in GC tissues, corresponding to the TNM stage of GC. We also found that GC patients with high MYPT1 expression had a longer survival time, and MYPT1 expression was higher in post-operative patients than in preoperative patients. In addition, the results indicated that overexpression of MYPT1 can suppress RhoA phosphorylation. In functional experiments, we have demonstrated that MYPT1 inhibits GC cell proliferation, migration, and invasion, and induces GC cell cycle arrest.

Previous studies have demonstrated that MYPT1 and CPI-17 can regulate basal LC20 phosphorylation in gastric fundus, murine gastric antrum, and proximal colon smooth muscles [56]; MYPT1 phosphorylation can regulate mammalian mitotic progression [57]; MYPT1 can affect contractility and microtubule acetylation to regulate matrix assembly and integrin adhesions [58]; MYPT1 degradation can promote the development of tolerance to nitric oxide in porcine pulmonary artery [59]; MYPT1 can affect vascular smooth muscle function and maintain blood pressure balance [60]; and phosphorylation of CPI-17 and MYPT1 can induce  $Ca^{2+}$  sensitization in intestinal smooth muscle [61]. In addition, a study showed that MYPT1 can promote the cycle progression of cancer cells [15]. In the present study, we found that MYPT1 expression is related to the development of GC; therefore, we suggest that MYPT1 might be a potential biomarker and therapeutic strategy for GC.

C-myc is an important regulation factor of the cell cycle, which is located in chromosome 8. C-myc can regulate many downstream genes, and then regulate the progression of cell cycle and apoptosis [62,63]. In the past few decades, it was found

that C-myc participates in the tumorigenesis of many malignant tumors [64]. In addition, C-myc is strictly regulated in normal cells and is out of control in tumor cells [65,66]. Cyclin Ds are the positive regulatory factors of the cell cycle, and can make cells access the S phase. A study proved that cyclin D1, which is a representative Cyclin D, has the most direct relationship with tumors. Overexpression of cyclin D1 can lead to G1 phase decrease and division speed acceleration [67]. In our study, we demonstrated that MYPT1 dramatically increased Cyclin D1 and c-myc expression, and our data indicate that MYPT1 inhibits GC cell proliferation and induces GC cell cycle arrest by Cyclin D1 and c-myc.

Matrix metalloproteinase (MMP) is secreted by connective tissue and is part of the extracellular matrix degradation  $Zn^{2+}$ -dependent protease family [68]. Studies showed that MMPs, such as MMP-2 and MMP-9, play major roles in the physiological and pathological processes of embryonic development, cell migration, angiogenesis, wound healing, atherosclerosis, malignant tumor infiltration, and metastasis [69–71]. Tissue inhibitor of metalloproteinases (TIMPs) is a set of low molecular weight glycoproteins that are widely distributed in tissues and fluids that can be produced and secreted by fibroblasts, epithelial cells, and endothelial cells. In addition, TIMPs are multifunctional proteins which can inhibit the activity of MMPs [72]. TIMPs participate in extracellular matrix remodeling and various pathological processes, such as tumor invasion, diffusion metastasis, and tissue fibrosis [73,74]. Previous research has shown that E-cadherin plays an important role in cellular adhesion in tumors, and its deletion was associated with tumor metastasis [75]. In our study, we found that MYPT1 increased E-cadherin expression and decreased TIMP-2 and MMP-2 expressions. The results indicate that MYPT1 inhibits GC cell migration and invasion by regulating E-cadherin, TIMP-2, and MMP-2.



## Conclusions

In summary, MYPT1 expression is associated with TNM stage, survival time, and prognosis of GC patients. In addition, MYPT1 inhibits GC cell proliferation, migration, and invasion via activating RhoA phosphorylation. This study identifies MYPT1 as a novel prognostic marker and candidate drug target for GC.

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## Conflict of interests

None.

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