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Association between *PRSS21* Expression and Prognostic Significance and Progression in Gastric Cancer

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Abstract

PRSS21, encoding testisin, has been reported to be related with some of tumor development and progression. However, little is known regarding the relation between *PRSS21* and gastric cancer (GC) progression. This study aimed to investigate the expression of *PRSS21* in GC and its relations with prognostic significance and progression of GC. The *PRSS21* expression in GC and normal tissues was compared by analyzing the data from The Cancer Genome Atlas (TCGA) datasets, meanwhile, its relationship with clinicopathological and prognostic significance (overall survival, OS) were determined by chi-squared test, Kaplan–Meier method and Cox proportional hazards model. The expression of *PRSS21* mRNA level in GC cells and normal gastric cells were tested by Quantitative real-time PCR (qPCR) assay. Knockdown of *PRSS21* was implemented by small interference (si-) RNA approach. Then, the function of *PRSS21* knockdown on GC cells proliferation and metastasis was assessed by Cell Counting Kit-8 (CCK-8), wound-healing and transwell assay, further, the markers of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling were measured by western blot assay. Results suggested that high-level of *PRSS21* was identified in GC tissues compared with normal gastric tissues. *PRSS21* overexpression associated with high Pathologic-Stage and shorter OS. Moreover, *PRSS21* expression level was higher in GC cells than that in normal gastric cells. Knockdown of *PRSS21* inhibited GC cells proliferation, migration and invasion, which was modulated by inactivation of PI3K/AKT signaling. Collectively, these findings shed some light on that *PRSS21* may be a potentially therapeutic target for GC treatment.

Keywords: *PRSS21*; Gastric cancer; Knockdown; Proliferation; Migration; Invasion

Introduction

Gastric cancer (GC) is one of the commonest deadly malignant types of gastrointestinal carcinoma. Notably, there are more than 680,000 new cases diagnosed and 500,000 deaths in China each year [1]. In spite of prominent improvements in detection and multimodal treatment, the survival rate of patients remains far from satisfactory [2,3]. The grim outcome is largely attributed to the lack of understanding of its pathogenesis that drives severe tumor stage and metastasis. Thereby, elucidation of the molecular mechanisms underlying GC to unearth appropriate prognostic biomarkers or therapeutic targets is imperative.

It is well known that serine proteases are involved in controlling proteolytic reactions responsible for various physiological and pathological processes, such as development, differentiation and tumorigenesis [4-6]. A testisin gene *PRSS21*, encoding a glycosylphosphatidylinositol-linked serine protease, is not expressed in testicular tumor cells while it is aberrantly expressed in some non-testis tumor cells [7]. It has been identified that *PRSS21* has a CpG island associated with methylation status of genes, which influences genes expression and even involves tumorigenic process. However, *PRSS21* is inactivated by DNA promoter hypermethylation in testicular tumor [8,9] Reports have indicated that testisin is highly expressed in cervical cancer cells, which promotes carcinogenesis by inhibiting tumor suppressor activity of maspin identified as a testisin-interacting molecule and a tumor suppressor protein [10-12]. There have studies demonstrating the regulatory role of serine proteases in angiogenesis, associated with tumor growth, being a hallmark tumor [13-15]. Nonetheless, little is known regarding the effect of *PRSS21* against GC progression so far.

In the current work, we analyzed the gene expression profiles and biological significance of *PRSS21* in GC found in The Cancer Genome Atlas (TCGA) database and uncovered that *PRSS21* expression was higher in GC tissues compared with normal tissues. Apart from this, highly expressed *PRSS21* was closely associated with clinicopathological significances and shorter overall survival (OS) of GC. Moreover, *PRSS21* upregulation was determined in GC cell lines in contrast with normal gastric cells. Finally, knockdown of *PRSS21* via small interference (si-) RNA approach impaired GC cells proliferation, migration and invasion, possibly by suppressing the PI3K/AKT signaling. In brief, the results of this study indicate that *PRSS21* may act as a tumorigenic action. Thereby, *PRSS21* would be considered as a potential therapeutic target for GC.

Materials and Methods

TCGA data mining and analysis

To determine the *PRSS21* expression and clinicopathologic implications in GC patients, we downloaded the enrolled GC samples and related clinicopathologic information from the TCGA database analyzed by limma package in R.

Cells culture

Human gastric cancer cell lines SGC -7901, MKN-45 and the normal human gastric mucosal cell line GES-1 were obtained from the Shanghai Institute for Life Science, Chinese Academy of Sciences

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(Shanghai, China). All cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). The cells were incubated in a humidified incubator with 5% CO_2 atmosphere at 37°C.

siRNA transfection

PRSS21 siRNA (si-*PRSS21*#1, 5'-ACUGACCUUAGUGAUCCCU-3'; si-*PRSS21*#2: 5'-UUCACCCUAUGACAUUGCC-3') and their negative control siRNA (si-NC: 5'-CCGUUACAGUAUCCCACUU-3') were purchased from Genechem (Shanghai, China). Lipofectamine 2000 reagent (Thermo Fisher Scientific) was applied to transfect cells following the manufacturer's protocols. Then, cells were incubated for 72 h and harvested for correlation analyses. The expression of *PRSS21* was confirmed by quantitative real-time PCR (qPCR) and western blot.

Quantitative real-time PCR (qPCR)

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's protocols. The first-strand cDNA was synthesized using a PrimeScript™ RT reagent kit (Takara Bio, Shiga, Japan) in accordance with the manufacturer's introductions. qPCR analysis of the mRNA expression of target genes was conducted using SYBR Premix Ex Taq™ kit (Takara) according to the manufacturer's instructions. GAPDH was used as an internal control. The primers used for PCR were: *PRSS21*: F: 5'-GCGCACTGCTTTGAAACTGA-3', R: 5'- AGG ATG GCA TGG AAG TCA GC-3'; GAPDH: F: 5'-GGA GCG AGA TCC CTC CAA AAT -3', R: 5'-GGC TGT TGT CAT ACT TCT CAT GG-3'. qPCR reaction conditions: 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. The comparative cycle threshold (Ct) method was used to quantify the expression levels by the $2^{-\Delta\Delta Ct}$ method. All experiments were carried out in triplicate.

Cell proliferation assay

Cell Counting Kit-8 (CCK-8) assay was performed to determine cell viability. Cells were plated in 96-well plates and cultured for 24, 48, 72 and 96 h. After incubation, 10 μl of CCK-8 reagent (Beyotime, Shanghai, China) was added to each well, and the plates were further incubated for 1 h in an incubator. The spectrophotometric absorbance (optical density, OD) at 450 nm was measured by a spectrophotometer (Bio-Rad, Hercules, CA, USA). Each experiment was performed in triplicate.

Wound healing and transwell assays

For wound healing assay, the cells were seeded in six-well plate and grown until reaching 90% confluence. Then, the confluence cells were scratched by a 200-μl pipette tip. After 24 h, the cells migrating into the wounded areas were observed and the wound width was photographed under a microscope at 100 × magnification. Next, the cell migration assay was executed using an 8-μm pore size transwell chambers (Corning, Lowell, MA, USA). At 48 h after transfection, 1×10^5 cells in 100 μl FBS-free medium were seeded into the upper chamber, and 500 μl culture medium containing 10% FBS was placed in the lower chamber. After 24 h incubation, non-migrated cells on the upper chamber were removed with cotton-tipped swabs, and those on the bottom surface were fixed with 4% paraformaldehyde for 30 min and then dyed with 0.1% crystal violet for 20 min. Finally, migrated cells were counted by photographing five randomly selected fields at 200 × magnification. Likewise, for the cell invasion assay, the protocols were alike with the cell migration assay, except that the transwell chambers were coated with Matrigel (Corning). Each experiment was performed in triplicate.

Western blot

After 48-h transfection, cells were lysed with Radio Immunoprecipitation Assay (RIPA) lysis buffer (Beyotime). The concentrations of protein were determined by BCA Protein Assay Kit (Beyotime). Equal amount (20 μg) of protein was separated on 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Thermo Fisher Scientific). After 1.5 h of blocking with 5% nonfat milk in TBST (Tris-buffered saline containing 0.1% Tween-20) at room temperature, membranes were incubated with primary antibodies, including phosphatidylinositol 3-kinase (PI3K), phosphorylated- (p-) PI3K, protein kinase B (AKT), p-AKT (Cell Signaling Technology, Danvers, MA, USA) at 1: 1000 dilution and Tubulin (Beyotime) at 1: 5000 dilution overnight at 4°C and with the anti-mouse/rabbit horseradish peroxidase-conjugated secondary antibodies (Beyotime, dilution 1: 1000) at room temperature for 2 h. Proteins were visualized by an enhanced chemiluminescence (ECL) detection system (Pierce Biotechnology, Inc., Rockford, IL, USA). Density of protein band was measured with Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

The association between clinicopathological features of the patients with GC and expression of *PRSS21* was analyzed using the chi-squared (χ^2) test. Overall survival (OS) curves were constructed using the Kaplan-Meier method. The distribution of survival was compared using the log-rank test. Univariate and multivariate analysis was performed using Cox's proportional hazard model. Statistical significance of difference between groups in experiments was analyzed using Student's t-test. For all statistical analyses, the SPSS system (version 22.0, SPSS INC., Chicago, IL) was used. The data were expressed as mean ± standard deviation (SD) and values of $p<0.05$ were considered as statistically significant.

Results

PRSS21 is highly expressed in the GC and correlates with clinicopathological characteristics

By analyzing data from TCGA datasets, we found that *PRSS21* expressions were significantly higher in GC tissues than in normal gastric tissues (Figure 1, p=1.88E-08). Further, we analyzed its relationship with clinicopathological features, such as Age, Gender,

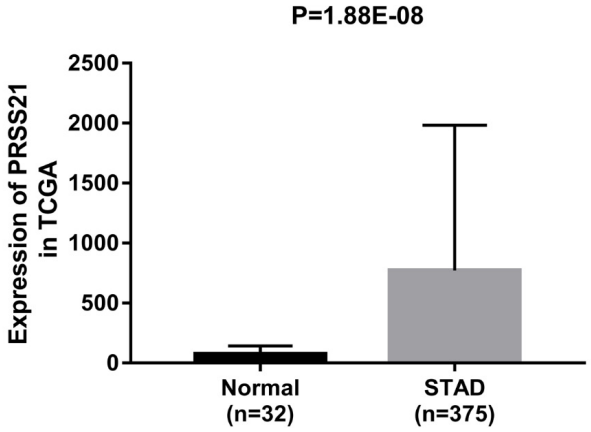

Figure 1: Highly expression of PRSS21 was identified in GC issues compared with normal gastric issues by analyzing the TCGA datasets. **p<0.01 versus normal gastric tissues.

Grade, Clinical-Stage, Tumor infiltration, Lymph node involvement, and distant metastasis. The data suggested that high *PRSS21* expressions were notably associated with high Pathologic-Stage (Table 1, p=0.019). Other clinicopathological parameters, including Age, Gender, Grade, Tumor infiltration, Lymph node involvement, and distant metastasis did not relate to *PRSS21* expression (all p>0.05). These results indicated that high *PRSS21* may be closely related with severe tumor stage of GC [16-19].

Correlation between *PRSS21* expression and prognosis

In the survival analysis, marked correlation was found between *PRSS21* expression and overall survival (OS) in patients. That is, high *PRSS21* expression in GC was correlated with shorter OS (Figure 2, p=0.028). Moreover, univariate Cox regression analysis revealed that Age, Clinical-Stage, Tumor infiltration, Lymph node involvement, distant metastasis and *PRSS21* expression predicted unfavorable OS of GC (Table 2, all p<0.05). In multivariate analyses, there was no relationship between *PRSS21* expression and OS, but Age and Distant metastasis were significantly associated with OS [20-26].

Elevated *PRSS21* expression in GC cells

mRNA level of *PRSS21* in GC cells and normal gastric cells were detected using qPCR. The data showed that *PRSS21* mRNA level was higher in both GC SGC -7901 and MKN-45 cells than in normal gastric GES-1 cells, particularly, this comparison in SGC-7901 was more obvious (Figure 3). Herein, SGC -7901 cells were applied in the following experiments.

PRSS21 knockdown inhibits the proliferation of GC cells

To examine the functional action of *PRSS21* on GC progression, silence of *PRSS21* using siRNA approach was introduced. The efficacy of silence was determined using qPCR and western blot assay. The results showed that *PRSS21* both in mRNA and protein was obviously decreased in si-*PRSS21*#2 group (more significant) or in si-*PRSS21*#1 group compared with si-NC group (Figure 4A and B, p<0.01). So, si- *PRSS21*#2 group was used in the following experiments and was presented as si-*PRSS21* group. Next, to determine the growth of GC cells after *PRSS21* knockdown, CCK-8 assay was performed. As showed in Figure 4, OD values in si- *PRSS21* group were reduced time-dependently compared with si-NC group (p<0.01). The results suggested that the proliferation of GC cells was inhibited by *PRSS21* knockdown.

Characteristics	Expression of PRSS21		*p*-value
	Low	High	
Age			0.195
<60	59	47	
≥60	113	122	
Gender			0.327
female	58	66	
male	115	105	
Grade			0.867
G1+G2	66	64	
G3	106	99	
Pathologic-Stage			0.019*
I+II	86	65	
III+IV	83	105	
Tumor infiltration			0.709
T1+T2	45	42	
T3+T4	125	128	
Lymph node involvement			0.071
N0	58	44	
N1+N2+N3	108	126	
Distant metastasis			0.162
M0	160	149	
M1	8	14	

Table 1: Association between clinicopathological parameters and PRSS21 expression in GC patients.

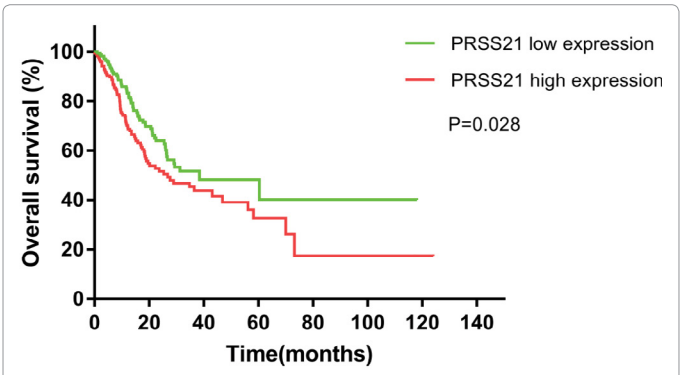

Figure 2: Overexpression of PRSS21 predicted a shorter OS in GC patient.

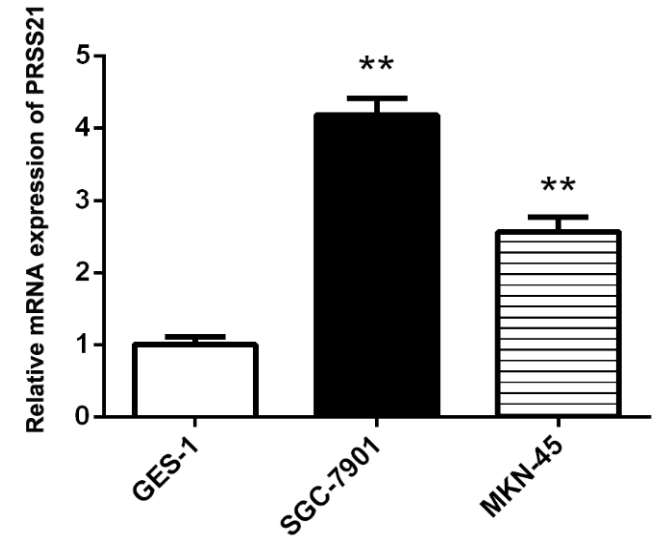

Figure 3: mRNA level of PRSS21 was higher in GC cells than in normal gastric cells. **p<0.01 versus normal gastric cells (GES-1).

PRSS21 knockdown suppresses the GC cells migration and invasion

To assess the effect of *PRSS21* knockdown on the properties of migration and invasion of GC cells, wound-healing and transwell assay was performed. As shown in Figure. 5A, delayed wound closure of SGC-7901 cells was observed in si-*PRSS21* group versus si-NC group by the wound-healing results. Next, the number of migrated and invaded cells was detectably decreased in si-*PRSS21* group on comparison with si-NC group (Figure. 5B, p<0.01). These data suggested that the abilities of migration and invasion of GC cells were inhibited by *PRSS21* knockdown (Figure 5).

PRSS21 knockdown triggers inactivity of PI3K/AKT signaling

PI3K/AKT signaling is known to play a distinctly critical role

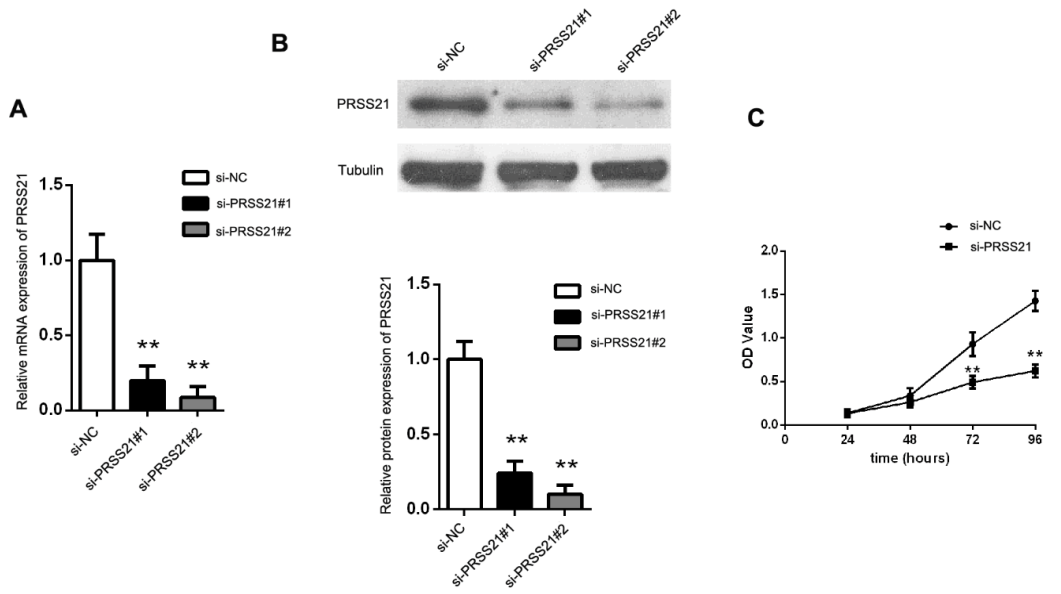

Figure 4: Knockdown of PRSS21 inhibited GC cell proliferation. A. The mRNA level of PRSS21 in GC cells after knockdown was determined by qPCR assay. B. The protein level of PRSS21 in GC cells after knockdown was determined by western blot assay. C. GC cells growth was assessed by measuring the OD values using the CCK-8 assay. ***p*<0.01 versus si-NC group.

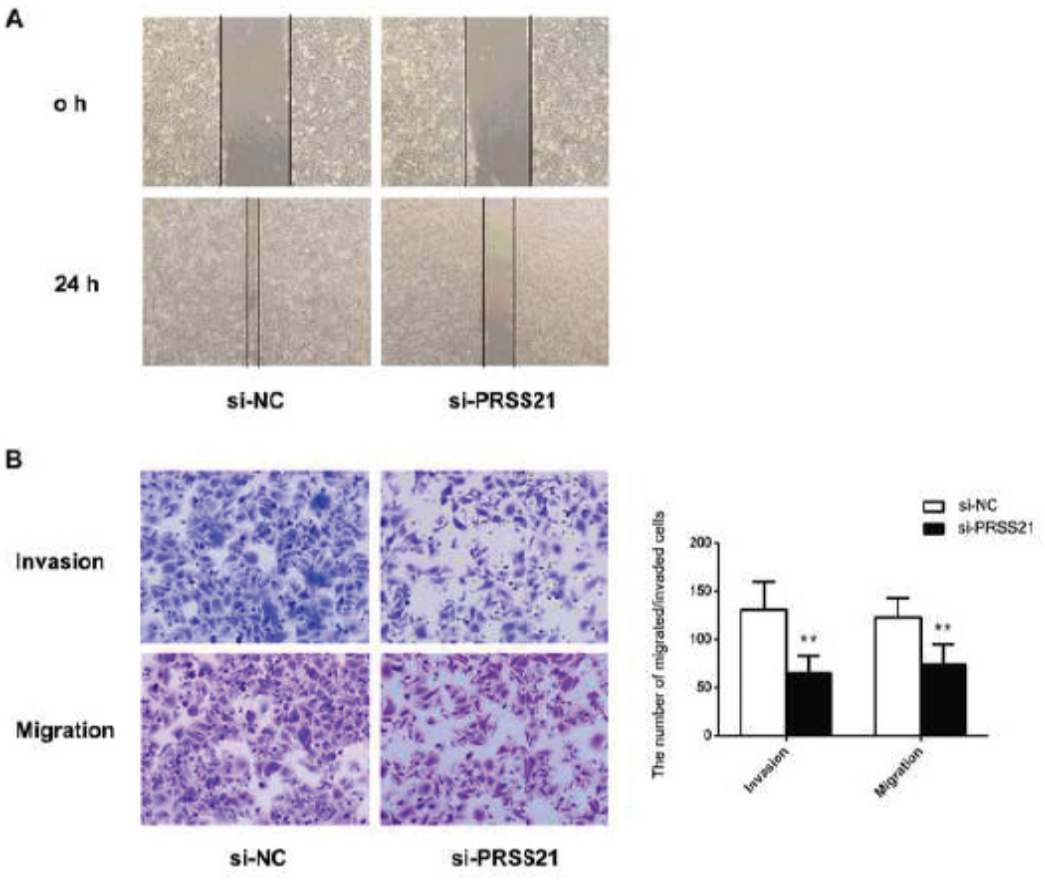

Figure 5: Knockdown of PRSS21 inhibited the mobility of GC cells. A. The mobility of GC cells was assessed by wound-healing assay. B. The abilities of migration and invasion in GC cells were measured by transwell assay. ***p*<0.01 versus si-NC group.

Variables	Univariate analysis			Multivariate analysis		
	p-value	HR	95%CI	*p*-value	HR	95%CI
PRSS21 expression (high/low)	0.029*	1.450	1.040-2.024	0.079	1.373	0.964-1.957
Pathologic-Stage (I+II/III+IV)	0.001*	1.890	1.320-2.705	0.527	1.186	0.699-2.012
Tumor infiltration (T1+T2/T3+T4)	0.021*	1.636	1.076-2.488	0.373	1.250	0.765-2.044
Distant metastasis (M0/M1)	0.004*	2.346	1.321-4.164	0.011*	2.182	1.192-3.994
Lymph node involvement (N0/N1+N2+N3)	0.003*	1.910	1.253-2.911	0.152	1.519	0.857-2.694
Age(<60/≥60)	0.033*	1.511	1.033-2.209	0.010*	1.697	1.133-2.541
Gender (Female/Male)	0.087	1.367	0.956-1.957	--	--	--
Grade(G1+G2/G3+G4)	0.128	1.312	0.925-1.861	--	--	--

Abbreviations: CI: Confidence Interval; HR: Hazard Ratio.
*Statistically significant.

Table 2: Analyses of prognostic variables for overall survival (OS) in GC using Cox proportional hazards regression.

in cancer initiation and progression. Thus, to gain insight into the mechanism of *PRSS21* knockdown on GC, we tested key markers of PI3K/AKT signaling, including the PI3K, p-PI3K, AKT and p-AKT using the western blot assay. The results showed that p-PI3K and p-AKT, but not PI3K and AKT, were apparently down-regulated in si-*PRSS21* group compared with si-NC group (Figure 6, $p<0.01$). Overall, these results provided evidence that the PI3K/AKT pathway was involved in *PRSS21*-induced oncogenic effect in GC cells.

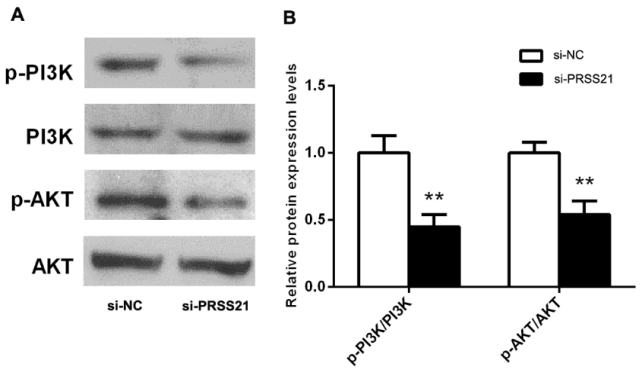

Figure 6: The activity of PI3K/AKT signaling was prohibited by knockdown of PRSS21. A. The key markers of PI3K/AKT signaling were tested by western blot assay. B. Densitometric quantification of protein bands were measured by Quantity One software. **$p<0.01$ versus si-NC group.

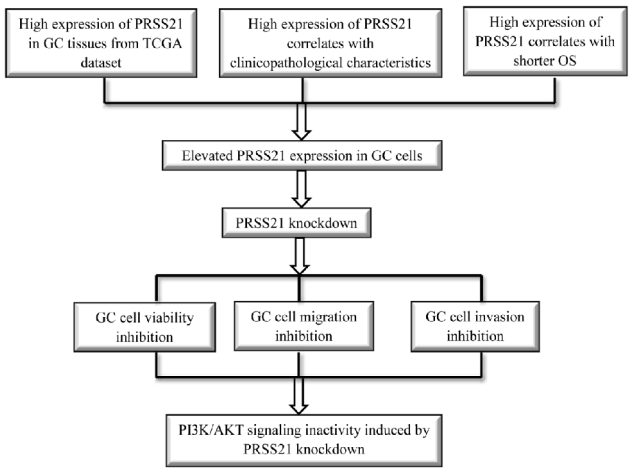

Figure 7: A schematic figure was exhibited to explain the whole findings.

Discussion

In the present study, high level of *PRSS21* was identified in GC samples in comparison with normal samples. *PRSS21* overexpression was correlated with high tumor stage and shorter OS in GC patients. Further, the biological effects of *PRSS21* knockdown in cancer cells were examined. We found *PRSS21* was higher in GC cells than normal gastric cells. In addition, knockdown of *PRSS21* impeded GC cells proliferation, migration and invasion through PI3K/AKT pathway. Overall, these findings imply that *PRSS21* might play an oncogenic function in GC and would be a promising therapeutic target in GC.

Modulation of serine proteases, a large multigene family, was of importance to tumor progression and development. It was found that the serine protease prostasin (PRSS8) was highly expressed in ovarian cancer samples compared with that in normal or benign ovarian lesions (Tamir *et al.*, 2016). Conversely, expression of PRSS8 was reported to be downregulated in colorectal or gastric adenocarcinomas and loss of it predicted a shorter OS of gastric patients (Bao *et al.*, 2016; Sakashita *et al.*, 2008). It was reported that loss of *PRSS21* expression was found in testicular tumors, thus *PRSS21* was previously proposed to be a tumor suppressor in testicular cancer (Hooper *et al.*, 1999). In contrast, *PRSS21* was found to be upregulated in cervical cancer (Shigemasa *et al.*, 2000). These findings indicated that *PRSS21* was associated with tumor progression. In this study, high-level of *PRSS21* was determined in GC samples or GC cells in comparison with normal samples or cells. In addition, overexpression of *PRSS21* predicted shorter OS of GC patients. Knockdown of *PRSS21* inhibited GC cells growth, migration and invasion. All these data implied that *PRSS21* might play an oncogenic function in GC progression.

It is well established that PI3K/AKT signaling plays a pivotal role of controlling many cellular functions, including cell growth, survival and migration (Engelman *et al.*, 2006). Of note, the key pathway was frequently altered in various types of cancers, including GC. Stimulated by growth factor receptor tyrosine kinases, the PI3K was activated, thus motivating the downstream activation of phosphoinositide-dependent kinase 1 (PDK1) and AKT, subsequently, mTOR complex 1 (mTORC1), which facilitates cell survival and cell growth (LoRusso, 2016). The inactivation of PI3K-AKT pathway by significantly decreased levels of p-PI3K and p-AKT expression was observed in SGC-7901 cells, which was involved in the anti-proliferation action and apoptosis initiation (Liu *et al.*, 2013). Increased Akt activation was reported to correlate with cell proliferation and survival, contrastively, the inhibition of Akt activation impaired the proliferation of GC cells

(Lee *et al.*, 2017). High p-AKT expression was found in 90% of GC while in 10% normal gastric tissues and overexpression of p-AKT was associated with gastric tumor size and presence of metastases (Chiappini *et al.*, 2017). Consistent with this, in this study, knockdown of *PRSS21* impeded the key molecules including p-PI3K and p-AKT, but not PI3K and AKT in GC cells. All data suggested that *PRSS21* may function as a carcinogenic role in GC possibly via PI3K/AKT signaling involvement. Nonetheless, our analysis existed some of limitations. First, clinical samples collected from our hospital as well as unfolding series of researches based on these samples were lack, which is the point of our following work. Second, the molecular mechanism underlying the oncogenic function of *PRSS21* was required to explore. To be more persuasive, animal model was put into effect in the further study. In spite of these challenges and limitations, this is the first investigation of assessing *PRSS21* expression in GC and preliminary anti-proliferative action of *PRSS21* knockdown *in vitro*.

Conclusion

In summary, experiments of our study demonstrated an increase in the expression of *PRSS21* in GC samples and its correlation with a more pathologic stage in GC patients. Additionally, the proliferation, migration and invasion abilities of GC cells were inhibited by *PRSS21* knockdown, which was probably regulated by PI3K/AKT signaling inactivation. The whole findings were explained in a schematic figure (Figure 7). Overall, our study suggests that *PRSS21* is likely to play promoting actions in carcinogenesis and progression of GC and may work as a valuable therapeutic target for treating GC patients in the future.

Declaration of Interest

The authors declare that they have no competing interests.

Authors' contributions

YDW designed the study and wrote the manuscript. CHH performed experiments and analyzed the data. All authors have read and agreed the final manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Consent to publish

All authors agree to the publication.

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