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ORIGINAL RESEARCH

G3BP1 activates the TGF-β/Smad signaling pathway to promote gastric cancer

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Background: GTPase-activating protein SH3 domain-binding protein 1 (G3BP1) is involved in various biological functions, including cell growth, metastasis, differentiation, apoptosis, and RNA metabolism. In current study, we aimed to investigate the effect of G3BP1 on gastric cancer (GC).

Methods: The expression of G3BP1 in GC tissues and cell lines was assessed by immunohistochemistry and Western blotting. Correlations of G3BP1 expression with clinicopathological and prognosis of GC patients were evaluated. The functions of G3BP1 in regulating proliferation, migration and invasion of GC cell were investigated using small interfering RNA (siRNA) strategies. Preliminary exploration of its underlying mechanism using Western blotting.

Results: G3BP1 expression was upregulated in GC tissues compared with adjacent tissues, and the higher G3BP1 expression was correlated with poor prognosis. G3BP1 knockdown decreased GC cell proliferation, migration and invasion. Mechanistically, silencing of G3BP1 inhibits the activation of the transforming growth factor (TGF)-β/Smad signaling pathway in GC cells.

Conclusion: G3BP1 plays an important role in the progression of GC as an oncogene and may become a new therapeutic target.

Keywords: gastric cancer, G3BP1, prognosis, TGF-β/Smad signaling pathway

Introduction

Gastric cancer (GC) is one of the most common malignancies of the digestive tract.[1] Although the incidence of GC has decreased in recent years, its mortality rate remains high. The incidence of GC in East Asia is the highest worldwide. Notably, China accounts for half of the world's GC patients.[2] Distant metastasis can even occur in the early stages of GC, while the 5-year survival rate of patients with advanced GC is under 30%.[3] Tumor progression involves activation of oncogenes and inactivation of tumor suppressor genes. Investigating the molecular mechanism of GC pathogenesis and its potential therapeutic targets is currently considered a research hotspot.

GTPase-activating protein SH3 domain-binding protein 1 (G3BP1), a RasGAP SH3 domain-binding protein belonging to the RNA-binding protein family, plays a crucial role in the regulation of Ras signaling.[4] G3BP1 is involved in various biological functions, including cell growth, metastasis, differentiation, apoptosis, and RNA metabolism. Multiple studies have shown that G3BP1 is closely associated with the progression of various solid malignancies; however, there are relatively few studies regarding G3BP1 in GC. Min et al found that G3BP1 is highly expressed in GC tissues, which is one of the poor prognostic factors for GC

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patients.[5] Nevertheless, the specific mechanism by which G3BP1 promotes GC remains unclear. In this study, we explored the relationship between G3BP1 and GC, and investigated the possible regulatory mechanisms.

Methods

Patients and specimens

In this study, cancerous and adjacent tissue samples from 120 GC patients who underwent surgery at Hubei Cancer Hospital from January 2010 to December 2013 were collected. Tissue samples were snap frozen in liquid nitrogen immediately after surgical resection and subsequently stored at –80 °C. Written informed consent was obtained from each patient before collecting their tissue samples. All patients were pathologically confirmed to have GC, and no radiotherapy, chemotherapy or immunotherapy was performed prior to surgery. GC staging was determined in accordance with guidelines from the Union for the International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC) TNM Staging System (8th Edition). This study was approved by the Ethics Committee of Hubei Cancer Hospital. The written informed consent was collected from all subjects and the study was conducted in accordance with the Declaration of Helsinki.

Cell culture and transfection

The GC cell lines AGS, MKN45, MGC-803, HGC-27, and SGC-7901 as well as normal gastric epithelial cell line GES-1 were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). The above-described cells were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 mg/mL of streptomycin. All cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was changed every three days. Cells were plated in 6-well plates at 60–70% confluence and small interfering RNA (siRNA) was used to suppress the expression of the gene of interest. The target sequence of G3BP1 was as follows: GGAGATTCATGCAAACGTT. Scrambled siRNA was used as a negative control. Transfection of siRNA was performed using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol.

Immunohistochemistry and evaluation

Paraffin-embedded tissue samples were consecutively cut into 4 μm sections, and then de-waxed using xylene and ethanol. The sections were placed in citrate solution for antigen retrieval and washed twice with phosphate buffered saline (PBS), followed by blocking endogenous peroxidase activity using 1.5% H_2O_2. After antigen retrieval, the sections were incubated with primary antibody against G3BP1 (13057-2-AP; Proteintech, Wuhan, China) at 4 °C overnight. After washing with PBS, the sections were incubated with secondary antibody for 30 mins at room temperature. Finally, the sections were stained with diaminobenzidine and counterstained with hematoxylin.

All sections were separately evaluated by two independent pathologists under a microscope. Based on the number of positive tumor cells, the staining score was as follows: <5% for 0, 5–24% for 1 point, 25–49% for 2 points, and 50–100% for 3 points. The staining intensity scores were as follows: blank (0), weak (1), medium (2), and strong (3). According to the percentage of positively stained tumor cells and staining intensity, a semi-quantitative classification score of RRBP1 protein expression level was used, where <4 indicated low expression and ≥4 indicated high expression.

Western blotting

Cells were lysed using RIPA buffer (Beyotime, Shanghai, China) for protein extraction, followed by measurement of protein concentration using BCA Protein Assay Kit (Beyotime). Cell lysates were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. After blocking with 5% nonfatty milk, the membrane was incubated overnight with anti-G3BP1 antibody (1:1000) at 4 °C. GAPDH (MultiSciences, Hangzhou, China) was used as an internal control. Subsequently, the membrane was incubated with goat anti-rabbit IgG secondary antibody (Thermo Fisher Scientific, USA). Other antibodies included: anti-TGF β1 antibody (1:2000), anti-TGF β2 antibody (1:1000), anti-Smad2 antibody (1:2000), anti-phospho-Smad2 (1:1000), anti-Smad3 antibody (1:1500), anti-phospho-Smad3 (1:2000). All the aforementioned antibodies were purchased from Abcam Inc. (Cambridge, MA, USA).

Cell counting kit-8 (CCK-8) assay

Cell viability was measured using CCK-8 (Dojindo Molecular Technologies, Japan). In brief, cells were placed into a 96-well plate at a density of about 2×10^3 cells/well. Then, 10 μL of CCK-8 reagent was added to each well and incubated at 37 °C for 1 hr. Absorbance was measured at 450 nm and at 24, 48, and 72 hrs using a Bio-Rad

Benchmark microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Transwell assay

Migration assay was performed using Transwell chamber (BD Biosciences, San Jose, CA, USA). Matrigel pre-coated Transwell chambers (BD Biosciences) were used for invasion assays. Briefly, 5×10^4 cells were resuspended in FBS-free medium in strict accordance with the manufacturer's instructions, and then subsequently placed in the Transwell chamber. Medium containing 10% FBS was added to the lower chamber. After culturing for an additional 24 hrs, cells in the upper chamber were wiped off, and cells in the lower chamber were fixed in 4% paraformaldehyde and then stained using 0.1% crystal violet.

Wound healing assay

Approximately 5×10^5 cells were seeded into 6-well plates and incubated to approximately 90–100% confluence. A 200 μL plastic pipette tip was used to create a uniform wound by gentle scraping. The wound healing process was monitored at 0 and 48 hrs using a microscope, and then photographed. Wound healing = (width at 0 h – width at 48 h)/width at 0×100%.

Statistical analysis

SPSS 19.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Chi-square test was used for comparison of categorical variables. Overall survival (OS) was used as an outcome indicator. Kaplan-Meier analysis was performed to determine survival, with log-rank test for comparison of patient survival between subgroups. The Cox proportional hazard model was utilized for univariate and multivariate analysis. All statistical analyses were two-sided and $P<0.05$ was considered statistically significant.

Results

G3BP1 expression in GC

We first evaluated the expression of G3BP1 in GC tissues. Using immunohistochemistry, we found that G3BP1 was localized in the cytoplasm of cells (Figure 1A and B). The expression of G3BP1 in GC tissues (81/120, 67.5%) was significantly higher than that in adjacent tissues (53/120, 44.2%; $P<0.01$). To further verify the above results, we randomly selected 30 cancer and adjacent tissue samples. Then, using Western blotting, we quantitatively detected the expression level of G3BP1 protein in the tissues. The results showed that G3BP1 content in GC tissues was significantly higher than that in adjacent tissues (Figure 1C). At the same time, we examined the expression level of G3BP1 in GC cell lines and normal gastric epithelial cell line. We found that G3BP1 expression level in the GC cell lines was higher than that in normal gastric epithelial cell line (Figure 1D).

Relationship between G3BP1 and prognosis of GC patients

According to the results of immunohistochemistry, 120 patients with GC were divided into G3BP1 high expression group (n=81) and low expression group (n=39). Next, we analyzed the relationship between G3BP1 expression status and clinicopathological features of GC patients. Among the variables, higher expression of G3BP1 was positively associated with tumor invasion, positive lymph node metastasis, and advanced TNM stages, but not associated with age, gender, tumor location, tumor differentiation, and Lauren classification. The above results are summarized in Table 1.

To investigate the relationship between G3BP1 and prognosis of GC patients, we further obtained the survival curves of patients with GC. Kaplan-Meier survival analysis shows that GC patients with high G3BP1 expression had shorter OS (Figure 2). We analyzed the value of G3BP1 in the prognosis of GC patients using Cox regression model. We identified Lauren classification, N classification, distant metastasis, TNM stage, and high G3BP1 expression as independent prognostic factors for OS in GC patients. The results of the single factor and multivariate analysis are shown in Table 2.

Silencing of G3BP1 inhibits the malignant behavior of GC cells

In previous studies, we found that G3BP1 protein has the highest expression level in the GC cell line MGC-803 (Figure 1D). Hence, we selected this cell line for all subsequent in vitro experiments. As shown in Figure 3A, G3BP1 was effectively knocked down by siRNA in MGC-803 cells. First, we assessed whether silencing of G3BP1 affects the proliferative capacity of MGC-803 cells. CCK-8 assay showed that silencing of G3BP1 significantly inhibited the proliferation of MGC-803 cells (Figure 3B). To further analyze the effect of G3BP1 on GC metastasis, we used cell migration assays to investigate the effect of G3BP1 silencing on MGC-803 cell metastatic ability. The results of Transwell migration

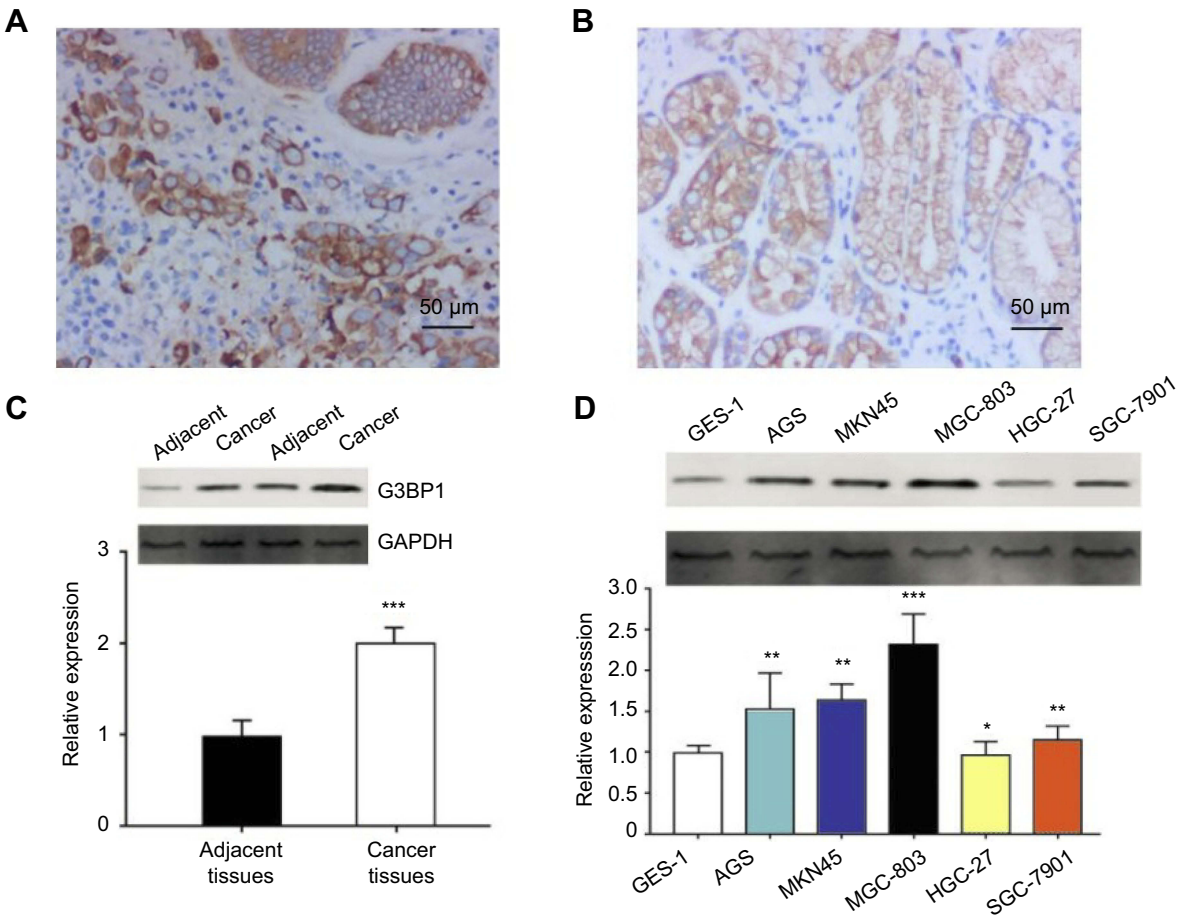

Figure 1 The G3BP1 protein expression in GC tissues and cell lines. The G3BP1 protein was observed in GC tissue (**A**) and adjacent tissue (**B**) by immunohistochemistry (×200), which is mainly located in the cytoplasm. (**C**) The G3BP1 protein was detected in GC tissues and adjacent tissues by Western blotting. (**D**) The G3BP1 protein was detected in GC cell lines by Western blotting. Compared with the adjacent group or GES-1 group, **P*>0.05; ***P*<0.05; ****P*<0.01.

assay indicated that silencing of G3BP1 inhibited the migration of MGC-803 cells (Figure 3C). The results of Matrigel invasion assay revealed that silencing of G3BP1 also inhibited the invasion of GC cells (Figure 3C). Wound healing assay is also an important method of assessing the migration ability of tumor cells. Consistent with the results of the Transwell migration assay, wound healing assay showed that knockdown of G3BP1 expression inhibited the motility of MGC-803 cells (Figure 3D).

Silencing of G3BP1 inhibits the activation of the transforming growth factor (TGF)-β/Smad signaling pathway in GC cells

Studies have shown that G3BP1 promotes tumor cell proliferation and metastasis by regulating the TGF-β/Smad signaling pathway.[6] However, the underlying regulatory mechanism in GC pathogenesis has not yet been investigated. Next, we investigated the effects of G3BP1 on TGF-β/Smad signaling. Western blotting results showed that G3BP1 knockdown inhibited the expression of TGF-β (TGF-β1 and TGF-β2) and the phosphorylation of Smad2 and Smad3 in MGC-803 cells (Figure 4). These results suggested that G3BP1 could control the activation of the TGF-β/Smad signaling pathway in GC.

Discussion

G3BP1 is a conserved, multifunctional protein containing two RNA-binding motifs in the C-terminal region.[7,8] G3BP1 exerts the role of endoribonuclease by selectively targeting genes combined with its consensus sequence.[9] The function of G3BP1 is regulated by RasGAP-dependent phosphorylation at serine 149.[9,10] Hyperphosphorylation of G3BP1 can maintain its endonuclease activity, whereas dephosphorylation facilitates the assembly of cytosolic protein-RNA aggregation, namely stress particles.[10] G3BP1 is a component of stress particles, which initiates the assembly

Table 1 Correlation between G3BP1 expression and clinicopathological characteristics in patients with GC

Parameters	No.	G3BP1		*P*-value
		High (*n*=81)	Low (*n*=39)	
Gender				0.581
Male	78	54 (66.7%)	24 (61.5%)	
Female	42	27 (33.3%)	15 (38.5%)	
Age				0.133
<60 y	58	43 (53.1%)	15 (38.5%)	
≥60 y	62	38 (46.9%)	24 (61.5%)	
Tumor location				0.721
Cardiac	19	14 (17.3%)	5 (12.8%)	
Body	30	21 (25.9%)	9 (23.1%)	
Pylorus	71	46 (56.8%)	25 (64.1%)	
Tumor differentiation				0.678
Well	10	8 (9.9%)	2 (5.1%)	
Moderate	18	12 (14.8%)	6 (15.4%)	
Poor	92	61 (75.3%)	31 (79.5%)	
Lauren classification				0.320
Intestinal Type	65	41 (50.6%)	24 (61.5%)	
Diffused Type	51	38 (46.9%)	13 (33.3%)	
Mixed Type	4	2 (2.5%)	2 (5.1%)	
T				**0.018**
T1+T2	39	32 (39.5%)	7 (17.9%)	
T3+T4	81	49 (60.5%)	32 (82.1%)	
N				**0.009**
N0	48	39 (48.1%)	9 (23.1%)	
N1-N3	72	42 (51.9%)	30 (76.9%)	
M				**0.016**
M0	99	63 (76.8%)	36 (94.7%)	
M1	21	19 (23.2%)	2 (5.3%)	
TNM stages				**0.005**
I-II	59	47 (58%)	12 (30.8%)	
III-IV	61	34 (42%)	27 (69.2%)	

Note: Bold indicates statistical significance at *P*<0.05.

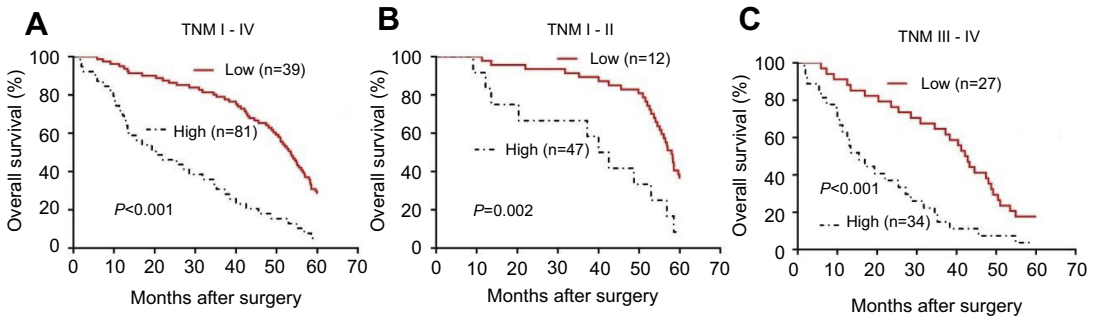

Figure 2 The relationship of G3BP1 protein expression status and OS of GC patients.

Table 2 Univariate and multivariate Cox regression analysis of clinicopathological characteristics influencing the overall survival of gastric cancer patients

Variables	Univariate		Multivariate	
	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
Gender Male vs female	1.049 (0.689–1.596)	0.825		
Age (Year) ≥60 vs <60	1.480 (0.984–2.228)	0.060		
Tumor location Cardia + body vs antrum	0.825 (0.636–1.070)	0.147		
Differentiation Poorly +moderately vs well	0.891 (0.649–1.222)	0.473		
Lauren classification Diffused +mixed vs intestinal	4.220 (2.710–6.570)	<0.001	4.196 (2.429–7.248)	<0.001
T classification T3-4 vs T1-2	4.918 (2.884–8.387)	<0.001	1.386 (0.851–2.257)	0.190
N classification N1-N3 vs N0	5.348 (3.324–8.605)	<0.001	2.411 (1.375–4.228)	0.002
M classification M1 vs M0	5.141 (2.000–13.218)	0.001	3.000 (1.131–7.954)	0.027
TNM stage III+IV vs I+II	2.783 (1.833–4.227)	<0.001	2.058 (1.135–3.734)	0.018
G3BP1 expression High vs low	3.185 (2.083–4.870)	<0.001	4.545 (2.673–7.728)	<0.001

of stress particles by forming multimers. Deletion of G3BP1 fails to cause eIF2α phosphorylation or eIF4A inhibition, and thus stress particles cannot be formed.[9,11,12] In recent years, certain studies have shown that G3BP1 also plays an important role in cancer progression.

Dou et al reported that G3BP1 expression is upregulated in hepatocellular carcinoma (HCC), and its high expression is significantly associated with poor prognosis in patients with HCC. G3BP1 is considered a carcinogen involved in the metastasis of HCC, which can regulate the expression of Slug.[13] In non-small cell lung cancer, miR-193A has been shown to promote metastasis of tumor cells by down-regulating G3BP1 expression.[14] Somasekharan[15] reported that YB-1 directly binds to and activates the 5′ untranslated region of G3BP1 mRNA, thereby controlling G3BP1 expression. The inactivation of YB-1 in human sarcoma cells significantly reduces G3BP1 expression in vitro. The expression of YB-1 and G3BP1 is highly correlated in human sarcoma, and elevated G3BP1 expression is associated with poor patient survival. Two existing studies on the relationship between G3BP1 and GC have shown that G3BP1 expression is up-regulated in GC.[5,16] In the present study, we also found that the expression level of G3BP1 in GC tissues was significantly higher than that in adjacent tissues. Similar to studies by Min et al,[5] we found that abnormal expression of G3BP1 is closely associated with tumor size, lymph node involvement, and TNM stage in patients with GC. G3BP1 is one of the factors predicting poor prognosis in GC patients.

In an in vitro assay, siRNA technology was used to effectively knock down the expression of G3BP1 in GC cell lines. Our findings confirmed the outcomes of the study by Min et al,[5] which reported that C3BP1 is a potent factor promoting the proliferation, migration, and invasion of GC cells. We focused on investigating the signaling pathway through which G3BP1 plays a role in malignancy. G3BP1 has been shown to promote tumor cell proliferation and metastasis and inhibit apoptosis by regulating the Ras, TGF-β/Smad, Src/FAK, and p53 signaling pathways.[6]

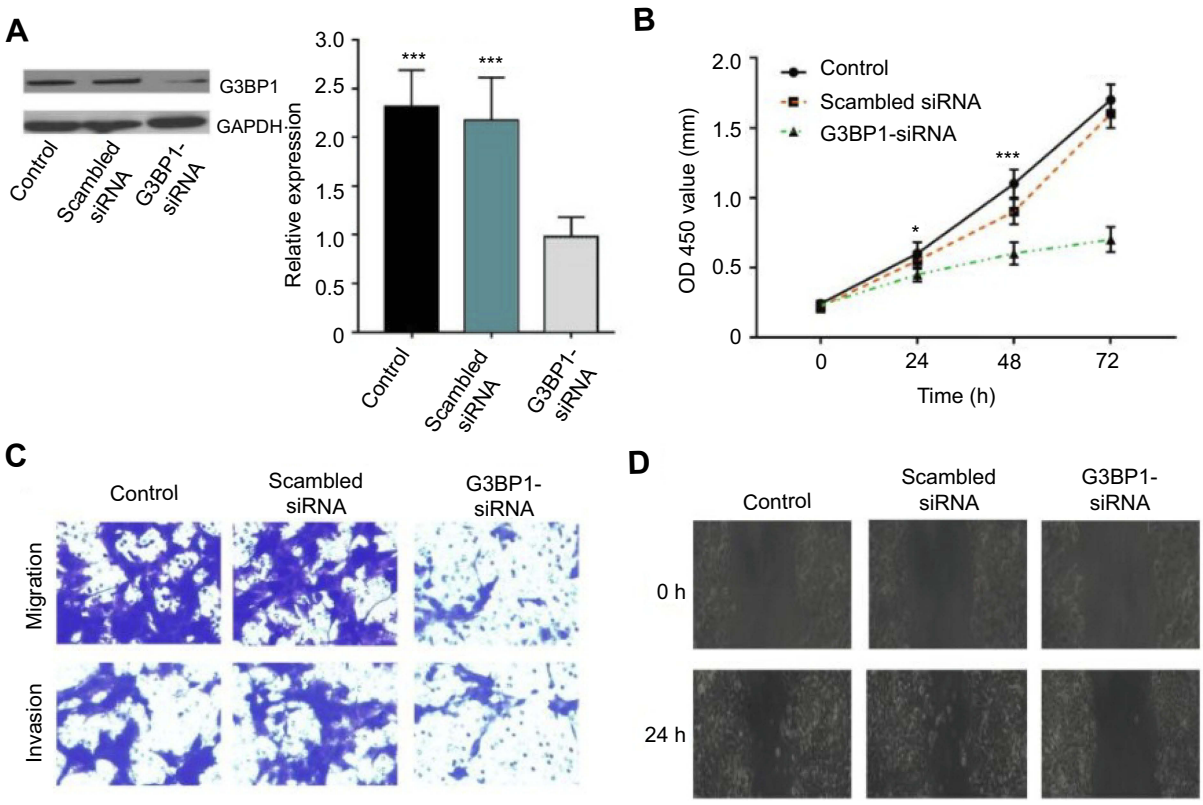

Figure 3 The relationship of G3BP1 protein expression status and malignant biological behavior of GC cell. (**A**) After transfection, the expression levels of G3BP1 protein in GC cells line was assessed by Western blotting. (**B**) CCK-8 assay showed that silencing G3BP1 expression can inhibit the proliferation of GC cells; (**C**) Transwell assay showed that silencing G3BP1 expression can inhibit the migration and invasion of GC cells. (**D**) Wound healing assay showed that silencing G3BP1 expression can inhibit the migration of GC cells. Compared with the G3BP1-siRNA group, **P*>0.05; ****P*<0.01.

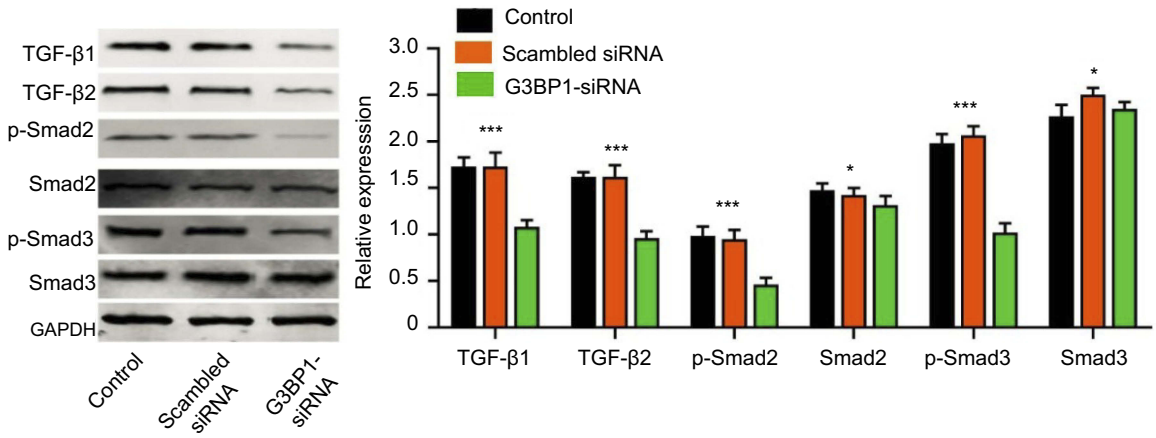

Figure 4 High G3BP1 expression activated TGF-β/Smad signaling pathway in GC cells. Compared with the G3BP1-siRNA group, **P*>0.05; ****P*<0.01.

Therefore, we detected the expression levels of TGF-β/Smad pathway-related molecules after G3BP1 knockdown in GC cell lines. The results preliminarily confirmed our hypothesis that G3BP1 plays a role in promoting cancer through the TGF-β/Smad signaling pathway.

In summary, G3BP1 is highly expressed in GC and is an independent risk factor for poor prognosis in GC patients. G3BP1 has a role in malignancy mainly through the TGF-β/Smad signaling pathway, which is a potential therapeutic target for GC. However, our understanding of

the regulatory mechanism of G3BP1 in GC is limited, and requires further exploration.

Disclosure

The authors report no conflicts of interest in this work.

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