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Basic Study

Ubiquitin-specific protease 15 contributes to gastric cancer progression by regulating the Wnt/ β -catenin signaling pathway

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Abstract

BACKGROUND

Ubiquitin-specific protease 15 (USP15) is an important member of the ubiquitin-specific protease family, the largest deubiquitinase subfamily, whose expression is dysregulated in many types of cancer. However, the biological function and the underlying mechanisms of USP15 in gastric cancer (GC) progression have not been elucidated.

AIM

To explore the biological role and underlying mechanisms of USP15 in GC progression.

METHODS

Bioinformatics databases and western blot analysis were utilized to determine the expression of USP15 in GC. Immunohistochemistry was performed to evaluate the correlation between USP15 expression and clinicopathological characteristics of patients with GC. A loss- and gain-of-function experiment was used to investigate the biological effects of USP15 on GC carcinogenesis. RNA sequencing, immunofluorescence, and western blotting were performed to explore the potential mechanism by which USP15 exerts its oncogenic functions.

RESULTS

USP15 was up-regulated in GC tissue and cell lines. The expression level of USP15 was positively correlated with clinical characteristics (tumor size, depth of invasion, lymph node involvement, tumor-node-metastasis stage, perineural invasion, and vascular invasion), and was related to poor prognosis. USP15

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knockdown significantly inhibited cell proliferation, invasion and epithelial-mesenchymal transition (EMT) of GC *in vitro*, while overexpression of USP15 promoted these processes. Knockdown of USP15 inhibited tumor growth *in vivo*. Mechanistically, RNA sequencing analysis showed that USP15 regulated the Wnt signaling pathway in GC. Western blotting confirmed that USP15 silencing led to significant down-regulation of β -catenin and Wnt/ β -catenin downstream genes (c-myc and cyclin D1), while overexpression of USP15 yielded an opposite result and USP15 mutation had no change. Immunofluorescence indicated that USP15 promoted nuclear translocation of β -catenin, suggesting activation of the Wnt/ β -catenin signaling pathway, which may be the critical mechanism promoting GC progression. Finally, rescue experiments showed that the effect of USP15 on gastric cancer progression was dependent on Wnt/ β -catenin pathway.

CONCLUSION

USP15 promotes cell proliferation, invasion and EMT progression of GC *via* regulating the Wnt/ β -catenin pathway, which suggests that USP15 is a novel potential therapeutic target for GC.

Key Words: Ubiquitin-specific protease 15; Gastric cancer; Wnt/ β -catenin; Cell proliferation; Cell invasion; Epithelial-mesenchymal transition

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Core Tip: Ubiquitin-specific protease 15 (USP15) was upregulated in gastric cancer (GC) cells and tissues, and was associated with a poor prognosis in patients with GC. USP15 promoted cell proliferation, invasion, and epithelial-mesenchymal transition of GC cells *in vitro* and tumor growth *in vivo*. Mechanistic studies showed that USP15 functioned as a tumor promoter in GC by regulating the Wnt/ β -catenin signaling pathway. Thus, USP15 is expected to be a novel potential target for GC therapy.

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INTRODUCTION

Gastric cancer (GC) has a high incidence worldwide and is one of the main causes of cancer-related deaths, especially in China[1,2]. Although there have been great advances in surgical procedures and targeted chemotherapy in recent years, the results are still not satisfactory and the survival rate is low, with median overall survival (OS) less than 12 mo[3-5]. Therefore, identifying novel potential targets for GC diagnosis and therapy and elucidating the underlying mechanisms of disease progression are essential for the prevention and treatment of GC.

In recent years, increasing evidence has shown that ubiquitin-specific proteases (USPs), the largest deubiquitinase subfamily, plays an important role in GC. For example, USP14[6], USP42[7], and USP44[8] are upregulated in GC and can be used as independent prognostic markers in GC patients. USP15, one of the most important members of the USP family, has been found to have some amplifications in many tumors. The N terminus of the protein encoding USP15 includes a ubiquitin-specific protease (DUSP) domain and two ubiquitin-like (UBL) domains, which can specifically remove the substrate protein by monoubiquitination and polyubiquitination modification[9]. The active site of the USP15 protein is located at Cys-269, and mutation of Cys269 to Ser (USP15 C269S) can inhibit enzyme activity[10]. Previous studies have reported that USP15 is upregulated in the liver and pancreatic cancer, and is associated with poor prognosis[11,12]. Mechanistically, USP15 can activate the transforming growth factor β (TGF- β) signaling pathway and promote the progression of advanced malignant glioma by combining the SMAD-specific E3 ubiquitin protein ligase 2

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complex and deubiquitinating and thus stabilizing the TGF β type I receptor[13]. In addition, USP15 can negatively regulate the function of p53 through affecting deubiquitination and stabilizing MDM2. Interestingly, inhibiting the activity of USP15 can induce tumor apoptosis and improve the antitumor T-cell response[14]. However, the role of USP15 in GC and its potential mechanisms have not been identified.

The Wnt/ β -catenin signaling pathway is involved in many cellular processes such as tumor growth, differentiation and invasion, and tumorigenesis[15]. It is often activated in many types of cancer, and the nuclear accumulation of β -catenin is an important sign of Wnt signaling activation[16]. The activation of β -catenin can activate many oncogenes including c-myc and cyclin D1, and regulate cell proliferation, cell cycle progression and apoptosis during tumorigenesis[17-19]. However, the mechanisms of Wnt/ β -catenin activation in GC have not been fully elucidated.

We found that USP15 was upregulated in GC cells and tissues, and was associated with a poor prognosis in GC patients. USP15 promoted cell proliferation, invasion, and epithelial-mesenchymal transition (EMT) of GC cells *in vitro* and tumor growth *in vivo*. Mechanistic studies showed that USP15 functioned as a tumor promoter in GC by regulating the Wnt/ β -catenin signaling pathway. Thus, USP15 is expected to be a novel potential target for GC therapy.

MATERIALS AND METHODS

Tissue samples

Paraffin-embedded GC samples, including cancerous tissues ($n = 115$) and adjacent tissues ($n = 30$), from May 2011 and May 2013, were obtained from the First Affiliated Hospital of Nanchang University (Nanchang, China). The clinicopathological characteristics of these patients are shown in Table 1. The fresh GC tissues ($n = 8$) and corresponding adjacent noncancerous tissues were stored in liquid nitrogen until use. This study obtained ethical approval from the Human Research Ethics Committee of the First Affiliated Hospital of Nanchang University.

Cell lines and culture

Human GC cell lines (SGC-7901, HGC-27, MKN-45, MGC-803, BGC-823, and AGS) and the human immortalized gastric epithelial cell line (GES-1) were purchased from the Beijing Beina Chuanglian Institute of Biotechnology (Beijing, China). The cells were cultured in (RPMI-1640) or Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS; HyClone, Logan, UT, United States) in an incubator with 5% CO_2 at 37 °C.

Immunohistochemistry

Immunostaining of USP15 proteins in 115 clinical GC samples followed previously described methods[20]. A primary antibody against USP15 (1:100, #66310; Cell Signaling Technology, Danvers, MA, United States) was used to detect the expression of USP15. All staining scores were evaluated blindly by two pathologists based on staining intensity and positive staining ratio. The grading standard of immunohistochemistry was carried out as previously described [20].

Cell Counting Kit-8 assay and colony formation assay

At 48 h after transfection, 2000 GC cells per well were seeded into a 96-well plate for the Cell Counting Kit-8 (CCK-8) assay and 1000 GC cells per well were seeded into a 6-well plate for the colony formation assay as previously described[21].

Wound healing assay

At 48 h after transfection, 5×10^5 GC cells per well were seeded into a 6-well plate, and the cells were starved for 24 h until complete fusion. Straight lines were drawn with a sterile 10- μL pipette tip to form wounds. Then the cells were carefully washed with phosphate-buffered saline (PBS) and cultured in serum-free medium. Images were captured at 0, 24, and 48 h to assess wound closure.

Transwell assay

The transwell assay was performed *via* using 8- μm transwell chambers (Merck KGaA, Darmstadt, Germany) with or without 60 μL Matrigel gel (BD Biosciences, Hercules, CA, United States), and then the chambers were put in each well of a 24-well plate. Cells of each group (5×10^4) were placed in 200 μL serum-free medium for 48 h after transfection, and subsequently transferred to the upper compartment of the above

Table 1 Ubiquitin-specific protease 15 expression and clinicopathological characteristics of gastric cancer patients

Parameters	<i>n</i>	USP15 expression		<i>P</i> value
		low	High	
Gender				
Male	62	20	42	0.218
Female	53	23	30	
Age in year				
≤ 60	53	18	35	0.482
> 60	62	25	37	
Differentiation				
Poor	69	25	44	0.753
Moderate/well	46	18	28	
Tumor size in cm				
≤ 4	66	32	34	0.004
> 4	49	11	38	
TNM stage				
I + II	58	28	30	0.015
III + IV	57	15	42	
Depth of invasion				
T1 + T2	54	27	27	0.009
T3 + T4	61	16	45	
LNI				
N0	43	24	19	0.002
N1 + N2+N3	72	19	53	
Perineural invasion				
No	51	25	26	0.021
Yes	64	18	46	
Vascular invasion				
No	50	27	23	0.001
Yes	65	16	49	
Total	115	43	72	

GC: Gastric cancer; LNI: Lymph node involvement; TNM: Tumor-node-metastasis; USP15: Ubiquitin-specific protease 15.

chambers. The lower chamber contained RPMI-1640 with 10% FBS. After 36 h of incubation, the cells that had migrated or invaded to the bottom side of the chamber were fixed with methanol, and then stained with crystal violet.

Immunofluorescence

We dipped the coverslip into the culture medium to allow the cells to attach and grow, and then washed the cells three times with PBS. At room temperature, the cells were fixed on a coverslip with 4% tetraformaldehyde for 20 min, and then were washed again three times with PBS. After a 10 min incubation with 0.5% Triton X-100, the cells were blocked in 5% bovine serum albumin for 2 h and then were incubated with anti-β-catenin antibody (1:200 dilution; Cell Signaling Technology) at 4 °C. After washing three times with PBS, cells were incubated with secondary antibody (1:50 dilution, ab150077; Abcam, Cambridge, MA, United States) for 1 h at room temperature. The coverslips were subsequently washed three times with PBS and then were stained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescence images were captured *via*

laser confocal microscopy.

Western blotting

Western blotting was performed following as previously described[22]. The following primary antibodies were used: USP15 (1:2000, #66310; Cell Signaling Technology), E-cadherin (1:1000, ab1416; Abcam), N-cadherin (1:1000, ab18203; Abcam), vimentin (1:1500, ab8978; Abcam), c-Myc (1:1500, #5605; Cell Signaling Technology), β -catenin (1:2000, #8480; Cell Signaling Technology), cyclin D1 (1:1000, #2978; Cell Signaling Technology), and GAPDH (1:2000, #60004-1-Ig; Proteintech, Rosemont, IL, United States).

Plasmid construction and cell transfection

To knock down the expression of USP15, three different small interfering RNAs (siRNAs) and a negative control (NC) were designed as followed: USP15-Homo-249, 5'-GGAACACCUUAUUGAUGAATT-3'; USP15-Homo-1150, 5'-GCAGAUGGAAGGC-CAGAUATT-3'; USP15-HoMo-1382, 5'-CCAAACCUAUGCAGUACAATT-3'; and a NC siRNA, 5'-UUCUCCGAACGUGUCACGUTT-3'. USP15 overexpression plasmid (USP15: NM_006313.2) and USP15 mutated plasmid (USP15-C269S) were based on pcDNA3.1 plasmid. The above siRNA and plasmid were synthesized by GenePharma (Suzhou, China). Cells were grown to 50%–60% confluency and transfected using TurboFect transfection reagent (R0532; Thermo Scientific Scientific, Waltham, MA, United States).

RNA sequencing analysis

The isolated USP15 knockdown and control BGC-823 cells were used for cDNA amplification and RNA sequencing (RNA-seq) library preparation. RNA-seq was performed by Beijing Novel Bioinformatics Co. Ltd. (Beijing, China). Genes with a false discovery rate < 5% and a fold change > 2.0 that met the established threshold criteria were considered to be significantly differentially expressed.

Nude mouse tumor cell xenograft assay

Short hairpin RNAs (shRNAs) targeting USP15 or scramble shRNAs were subcloned into the lentiviral expression vector (Jikai Co. Shanghai, China). BGC-823 cells transfected with LV-shUSP15 or LV-scramble shRNA was stably expressed and screened by puromycin. The stably expressed strain was amplified and inoculated at a rate of 5×10^6 cells per animal into 5- to 6-wk-old BALB/c-nu mice. Tumor volume was measured every 3 d and calculated according to the formula: volume (mm^3) = (length \times width²)/2. Mice were sacrificed after 28 d and xenograft tumors were measured and weighed. Proteins were extracted from tumors and USP15 and β -catenin expression was detected by western blotting.

Statistical analyses

We statistically analyzed the data using SPSS version 26.0 software (Chicago, IL, United States). The relationship between clinical characteristics and USP15 expression was evaluated by the χ^2 test. The Kaplan–Meier method was performed to determine the OS curve of all enrolled GC patients. Student's *t*-test was used to determine the mean difference between two groups. *P* < 0.05 was considered statistically significant.

RESULTS

USP15 is upregulated in GC cells and tissues and is associated with a poor prognosis in patients with GC

First, an online database, cBioPortal for Cancer Genomics (<http://www.cbioportal.org/>)[23], showed that USP15 was amplified in many types of tumor including GC (Figure 1A). TIMER (<https://cistrome.shinyapps.io/timer/>)[24] and UALCAN database (<http://ualcan.path.uab.edu/>)[25], based on The Cancer Genome Atlas (TCGA) database, indicated that the mRNA levels of USP15 in GC tissues were higher than those in normal tissues (Figure 1B and C). To confirm the protein expression level of USP15 in GC, western blotting was conducted on GC cell lines and tissues. Most GC cell lines expressed a higher level of USP15 than the gastric epithelial cell line GES-1 (Figure 1D). USP15 was elevated in most of the eight pairs of clinical GC tissues and their adjacent normal tissues (Figure 1E).

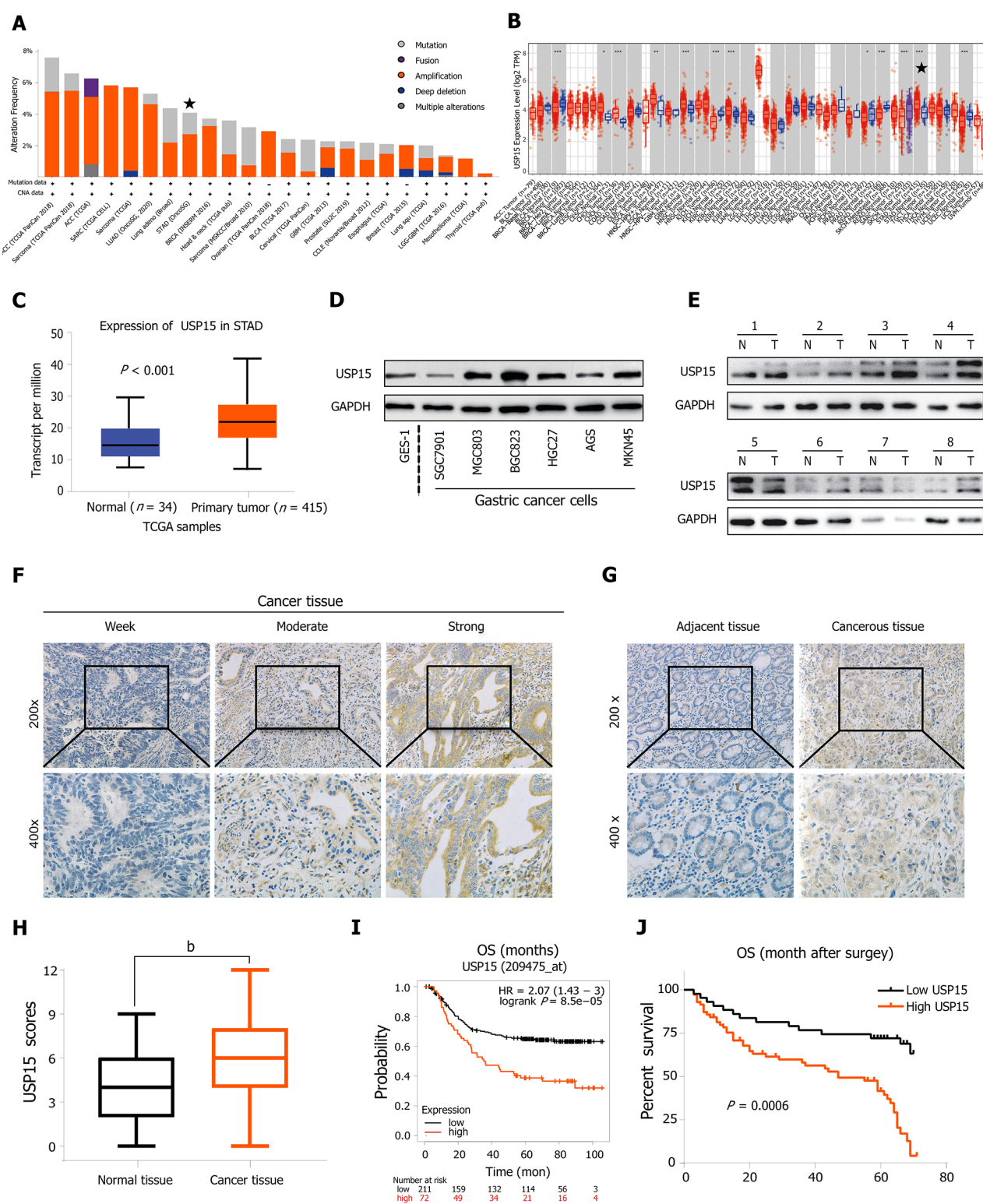


Figure 1 Ubiquitin-specific protease 15 is upregulated in gastric cancer cells and tissues, and is associated with a poor prognosis in gastric cancer patients. A: Frequency of ubiquitin-specific protease 15 (USP15) in various cancers, including gastric cancer (GC). The data were derived from the cBioPortal for Cancer Genomics; B, C: USP15 mRNA expression level of cancer tissue and normal tissue in GC. Analysis of The Cancer Genome Atlas data from TIMER and UALCAN databases; D: Protein expression of USP15 in GES-1 cell line and GC cell lines; E: Protein expression of USP15 in GC tissue and adjacent noncancerous tissues ($n = 8$); F, G: Representative images of immunohistochemical staining of USP15 in human GC tissues and adjacent noncancerous tissues; H: USP15 staining scores of GC and normal tissues; I: Correlation between USP15 expression level and overall survival (OS) in GC patients obtained from Kaplan-Meier Plotter databases (209475_at, HR = 2.07 (1.43-3), log rank $P = 8.5e-05$); J: Kaplan-Meier curve stratified by USP15 expression in 115 GC patients (log-rank test, $P = 0.0006$). ^a $P < 0.01$, data were expressed as the mean \pm standard error of the mean.

As USP15 was found to be upregulated in GC, we confirmed its clinical significance *via* using immunohistochemistry. The staining of USP15 protein ranged from weak to strong and located in the cytoplasm (Figure 1F), which showed that USP15 was markedly increased in GC tissue sections, whereas USP15 staining was weak or negative in noncancerous tissue sections (Figure 1G). The staining scores of USP15 in adjacent tissues were significantly lower than those in GC tissues, which were considered significantly different (Figure 1H).

Subsequently, we evaluated the correlation between the staining score of USP15 and the clinicopathological characteristics of patients. There was no significant difference among patient gender, age, differentiation, and USP15 expression; however, tumor size ($P = 0.004$), tumor-node-metastasis (TNM) stage ($P = 0.015$), depth of invasion ($P = 0.009$), lymph node involvement (LNI) ($P = 0.002$), perineural invasion ($P = 0.021$), and vascular invasion ($P = 0.001$) were significantly associated with USP15 expression in GC. Consistent with the results obtained from Kaplan–Meier Plotter Database Analysis[26] (<http://kmplot.com/analysis/>), the Kaplan–Meier curve stratified by USP15 expression in these 115 GC patients showed that patients with lower USP15 expression had longer OS (Figure 1I and J).

Knockdown of USP15 inhibits cell proliferation, invasion, and EMT progression of GC *in vitro*

As shown above, BGC-823 and MKN-45 cells had high expression of USP15. siRNA-mediated knockdown of USP15 expression in BGC-823 and MKN-45 cells was used to detect the function of USP15 *in vitro*. Western blotting was used to confirm the silencing efficiency of USP15 in GC cells (Figure 2A). The results of CCK-8 and colony formation assays showed that the proliferation rate and colony formation ability were markedly decreased in the USP15-siRNA-1/2 group compared to the NC group (Figure 2B and C). Based on correlation of USP15 expression and lymph node status, perineural and vascular invasion, wound healing and transwell assays were used to evaluate the role of USP15 in tumor cell migration and invasion. As shown in Figure 2D and Figure 2E, USP15 silencing suppressed GC cell migration and invasion. In addition, knockdown of USP15 upregulated E-cadherin and downregulated N-cadherin and vimentin (Figure 2F).

USP15 overexpression promotes cell proliferation, invasion, and EMT progression in GC

We explored the cellular behavioral changes caused by overexpression of USP15. A stably transfected cell line with USP15 overexpression plasmid, USP15 mutant plasmid (USP15-C269S), and a NC (empty-vector) cell line were established in SGC7901 cells. Western blotting confirmed the transfection efficiency (Figure 3A). Compared with the empty vector group, proliferation of the USP15 group was significantly enhanced, while the USP15-C269S group had no changes (Figure 3B and C). Overexpression of USP15 promoted GC cell migration and invasion, while USP15-C269S did not (Figure 3D and E). Western blotting analysis showed that overexpression of USP15 upregulated vimentin and N-cadherin but downregulated E-cadherin (Figure 3F). Collectively, these data demonstrated that USP15 overexpression promoted GC proliferation, invasion, and EMT progression.

USP15 regulates the Wnt/ β -catenin pathway in GC cells

To explore the potential molecular mechanism responsible for the effects of USP15 on GC progression, the whole transcriptome profiles of BGC-823 cells with USP15 knockdown or NC were analyzed by RNA-seq. The transfection efficiency was confirmed by western blotting (Figure 4A). The most differentially expressed genes (DEGs) (29829) were displayed on the heat map (Figure 4B). Among the 2343 significant DEGs (adjusted $P < 0.05$), transcripts of 1134 genes were upregulated and transcripts of 1209 were downregulated in USP15 knockdown groups compared to the control groups (Figure 4C). Gene Ontology enrichment analyses showed that the difference in Wnt signaling pathway was the most obvious (Figure 4D) among the enriched pathways.

As one of the most classic Wnt signaling pathways, the Wnt/ β -catenin pathway has been involved in multiple physiological processes of GC progression. To confirm the role of the Wnt/ β -catenin signaling pathway in the malignant biological behavior in GC mediated by USP15, western blotting was performed to investigate expression of β -catenin and Wnt/ β -catenin downstream genes (including c-myc and cyclin D1). USP15 knockdown resulted in downregulation of the protein level of β -catenin, c-Myc and cyclin D1, while USP15 overexpression yielded opposite results and there was no

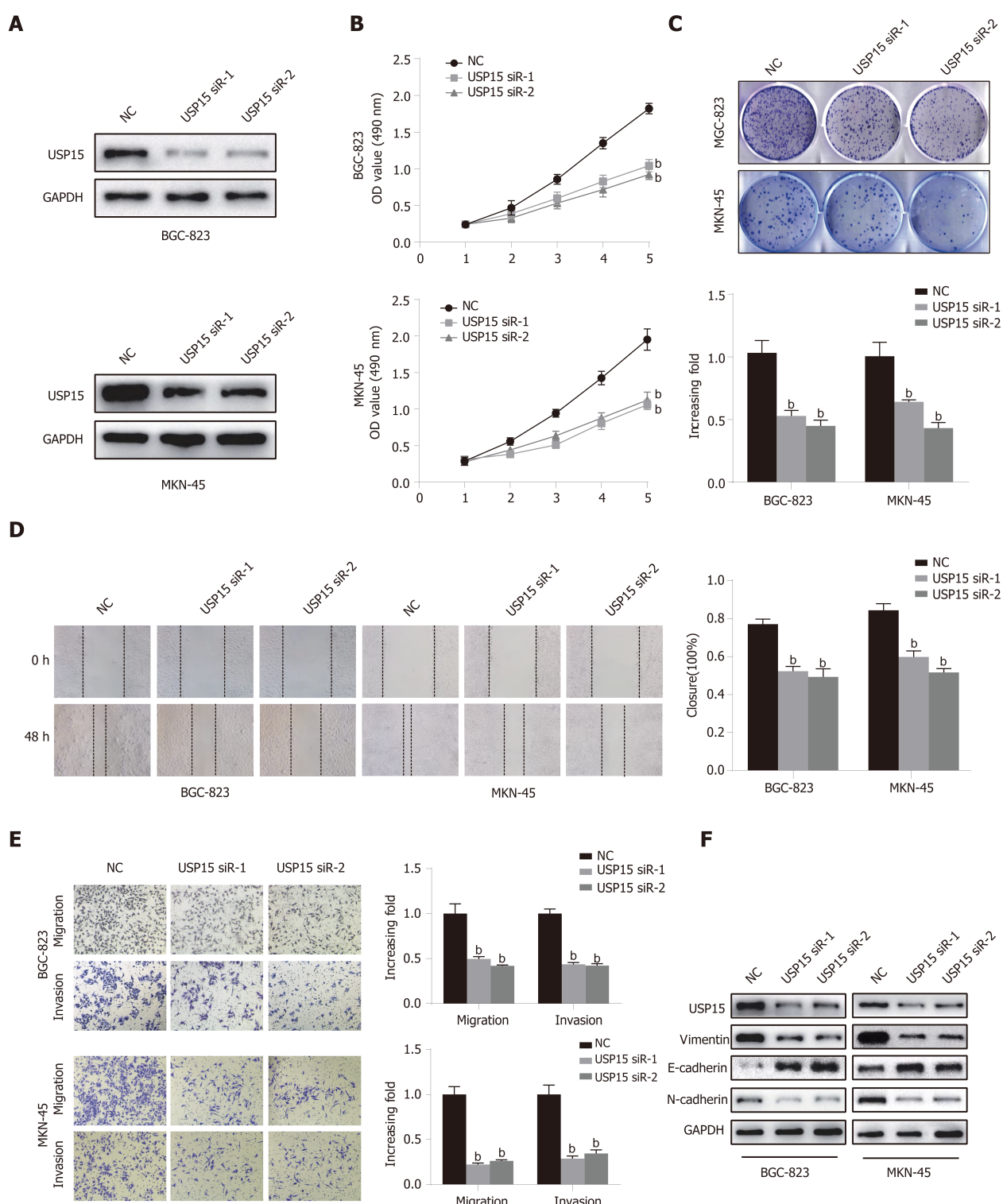


Figure 2 Knockdown of ubiquitin-specific protease 15 inhibits cell proliferation, invasion, and epithelial-mesenchymal transition progression of gastric cancer *in vitro*. A: BGC-823 and MKN-45 cells were transfected with ubiquitin-specific protease 15 (USP15) small interfering RNA or negative control for 48 h, and the efficiency was detected by Western blotting; B and C: Cell Counting Kit-8 assay and colony formation assay evaluated cell proliferation ability; D and E: Wound healing assay and transwell assays evaluated migration and invasion; F: Western blotting detected epithelial-mesenchymal transition markers (E-cadherin, vimentin, and N-cadherin). ^b $P < 0.01$, data were expressed as mean \pm standard error of the mean.

change in USP15 C269S group (Figure 4E). In addition, immunofluorescence assay showed that USP15 knockdown significantly reduced nuclear β -catenin accumulation compared with the control groups, while USP15 overexpression yielded opposite results, and there was no change in the USP15 C269S group (Figure 4F).

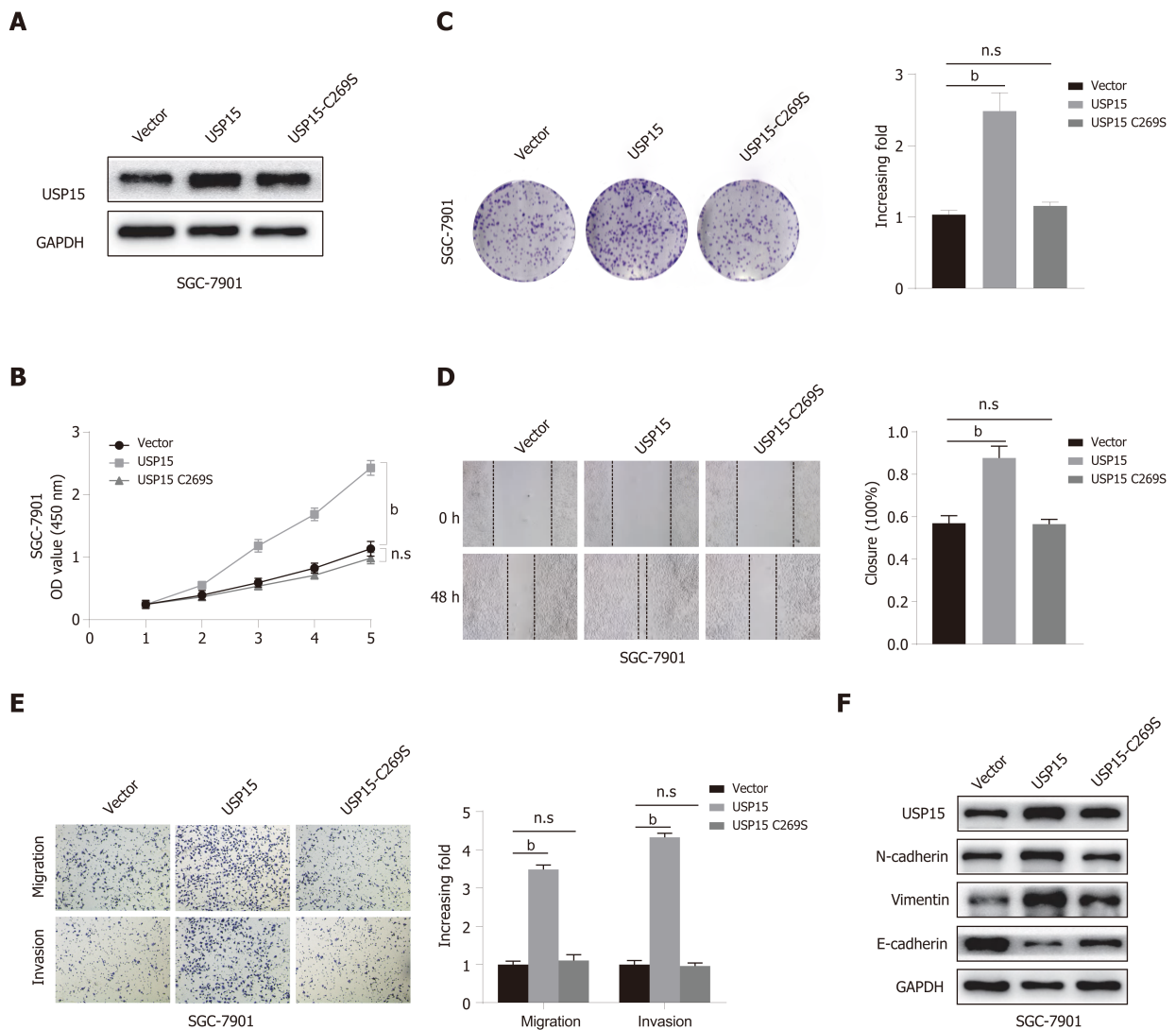


Figure 3 Overexpression of ubiquitin-specific protease 15 promotes proliferation, invasion, and epithelial-mesenchymal transition of gastric cancer cells. A: SGC-7901 cells were transfected with ubiquitin-specific protease 15 (USP15) overexpression plasmid, USP15-C269S plasmid (USP15 mutant) or empty vector for 48 h, and the efficiency was detected by Western blotting; B, C: Cell Counting Kit-8 assay and colony formation assay evaluated cell proliferation ability; D, E: Wound healing assay and transwell assay evaluated migration and invasion; F: Western blotting detected epithelial-mesenchymal transition markers (E-cadherin, vimentin, and N-cadherin). ^b*P* < 0.01, data are expressed as the mean ± standard error of the mean. n.s.: Not significant.

Lithium chloride partly reversed the effects of USP15 knockdown on GC progression

To further clarify whether the function of USP15 in GC was mediated by the Wnt/ β -catenin pathway, we performed a rescue experiment using lithium chloride (LiCl) (Wnt/ β -catenin pathway activator). The cell proliferation ability of BGC-823 and MKN-45 cells transfected with USP15 siRNA-1 was significantly elevated after treatment with LiCl compared to the untreated group (Figure 5A and B). Furthermore, the inhibition of invasion by USP15 knockdown can also be partly reversed by LiCl (Figure 5C). In addition, LiCl-treatment induced upregulation of β -catenin, c-myc, and cyclin D1 (Figure 5D). The above findings suggest that the function of USP15 on GC progression is dependent on Wnt/ β -catenin pathway.

USP15 knockdown inhibits tumor growth in vivo

We investigated the function of USP15 *in vivo*. BGC-823 cells transfected with LV-shUSP15 or LV-scramble shRNA were subcutaneously injected into nude mice to establish a xenograft mouse model. After the mice were sacrificed on day 29, we obtained tumor images (Figure 6A). Compared with the scramble shRNA group, USP15 knockdown reduced tumor volume and weight (Figure 6B and C). In addition, USP15 knockdown significantly reduced the protein levels of β -catenin, c-myc, and cyclin D1 in tumor tissue of nude mouse, consistent with the *in vitro* results.

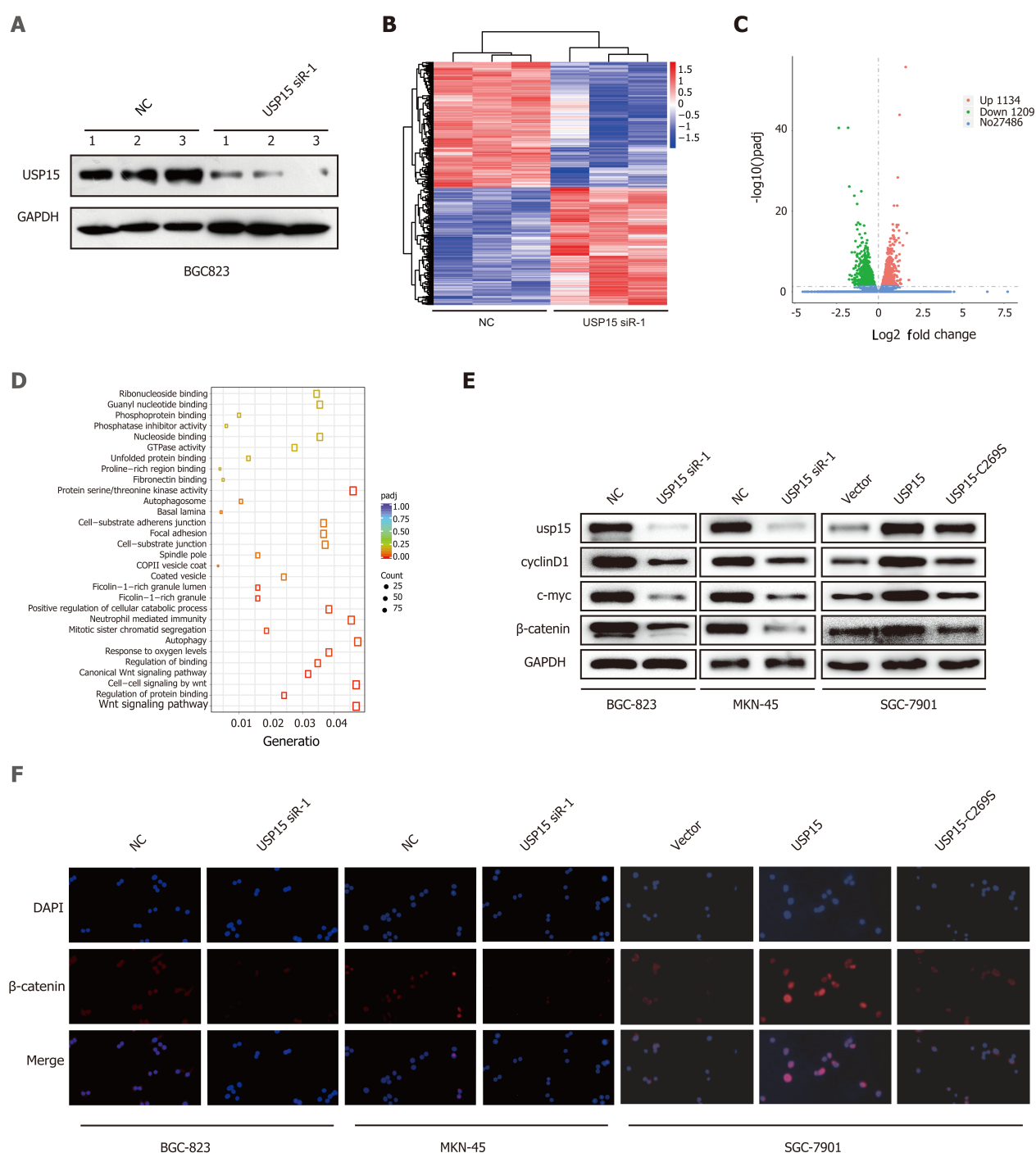


Figure 4 Ubiquitin-specific protease 15 regulates the Wnt/β-catenin pathway in gastric cancer. A: Western blotting confirmed the transfection efficiency of ubiquitin-specific protease 15 (USP15) knockdown in BGC-823 cell lines before RNA sequencing analysis; B: Heat map and hierarchical clustering based on the most differentially expressed genes (5739); C: Volcano plot illustrated differentially regulated gene expression. 1134 upregulated genes (red) and 1209 downregulated genes (green); D: Gene Ontology enrichment analyses of targets associated with USP15 for biological process, cellular component, and molecular function (top 10 most significantly affected categories are shown); E: Expression levels of USP15, β-catenin, c-Myc, and cyclin D1 in BGC-823 and MKN-45 cell lines transfected with USP15 knockdown or SGC-7901 cell lines transfected with USP15 overexpression; F: Immunofluorescence staining of β-catenin in BGC-823 and MKN-45 cell lines transfected with USP15 knockdown. Blue: DAPI; Red: β-catenin. Bar = 50 μm.

(Figure 6D).

DISCUSSION

In recent years, an increasing number of USP proteins have been reported to be critical to human cancers. For example, high expression of USP28 is related to the OS of patients with non-small cell lung cancer[27], while the expression of USP22 and USP11

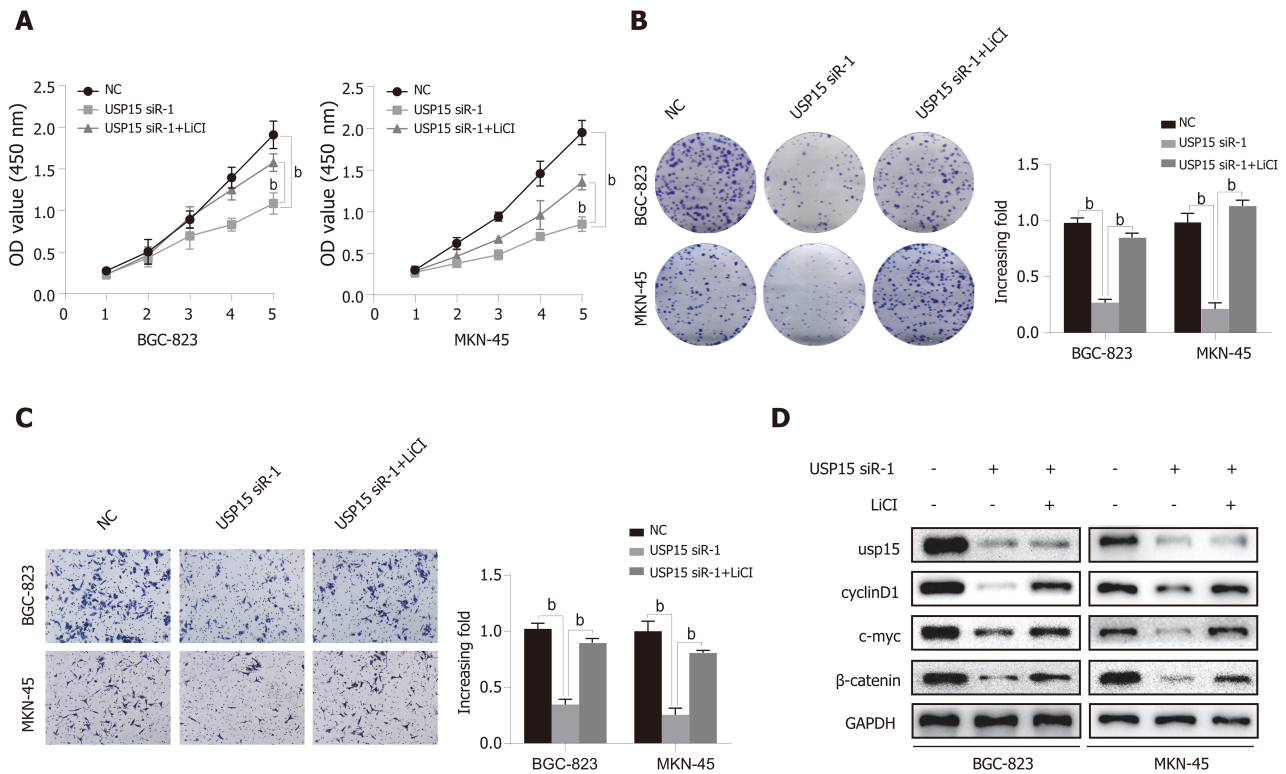


Figure 5 Lithium chloride partly reverses the effects of ubiquitin-specific protease 15 knockdown on gastric cancer progression. BGC-823 and MKN-45 cells transfected with ubiquitin-specific protease 15 (USP15) small interfering RNA-1 or negative control were incubated with or without lithium chloride (LiCl) (20 mmol/L). A, B: The Cell Counting Kit-8 (CCK-8) assay and colony formation assay evaluated cell proliferation ability; C: Transwell assays evaluated cell invasion ability; D: Western blotting detected the protein expression of β -catenin, c-Myc, and cyclin D1. $^bP < 0.01$, data are expressed as the mean \pm standard error of the mean.

is related to the poor prognosis of breast cancer[28,29]. Two recent studies have shown that USP15 is upregulated in liver cancer and pancreatic ductal cell carcinoma[11,12]. In this study, IHC analyses showed that the high expression of USP15 was closely related to the depth of invasion, LNI, TNM stage, which indicated that USP15 acted as an oncogene, thereby promoting GC invasion, metastasis, and progression. In addition, the high expression of USP15 was related to the poor survival rate of GC patients, suggesting that USP15 is very important in the pathogenesis and development of GC, and could be used as a prognostic biomarker.

Similar to previous results[11,12], our study confirmed that USP15 was significantly associated with tumor cell proliferation *in vitro*. In addition, we also found that USP15 could participate in the tumor growth *in vivo*. Subsequently, we further found that USP15 can significantly promote the migration and invasion of GC cells *in vitro*. Migration and invasion, as the basic characteristics of malignant tumors, are the main reasons for the short survival time of cancer patients[30,31]. Mounting evidence has shown that tumor cells after EMT has high motility and aggressiveness, among which E-cadherin, N-cadherin, and vimentin are important molecular markers[32]. In addition, the epithelial marker E-cadherin is downregulated, while the mesenchymal markers vimentin and N-cadherin are upregulated during EMT[32]. As shown in our results, knockdown of USP15 resulted in upregulation of E-cadherin and downregulation of N-cadherin and vimentin, while overexpression of USP15 had the opposite effects, suggesting that USP15 can induce EMT in GC cells. The above findings indicated that USP15 may promote cell proliferation, migration, invasion and EMT process to become an oncogene of GC.

USP15 was related to a variety of cell signaling events, including transforming growth factor β (TGF- β)[13,33], constitutive photomorphogenesis 9 (COP9) signaling body[34], p53 signaling pathway[14], and nuclear factor kappa B (NF- κ B)[35]. For example, USP15 promotes the stabilization of TGF- β receptor and its downstream signal transducers, thereby resulting in enhanced TGF- β signaling[13,36]. USP15 can protect the constituent subunits of cullin-RING ubiquitin ligase from self-ubiquitination and degradation *via* a stable cooperation with COP9-signalosome[34,37]. USP15 can stabilize MDM2 and negatively regulate the protein level of p53, and inactivation of USP15 can induce tumor apoptosis and improve the antitumor T-cell

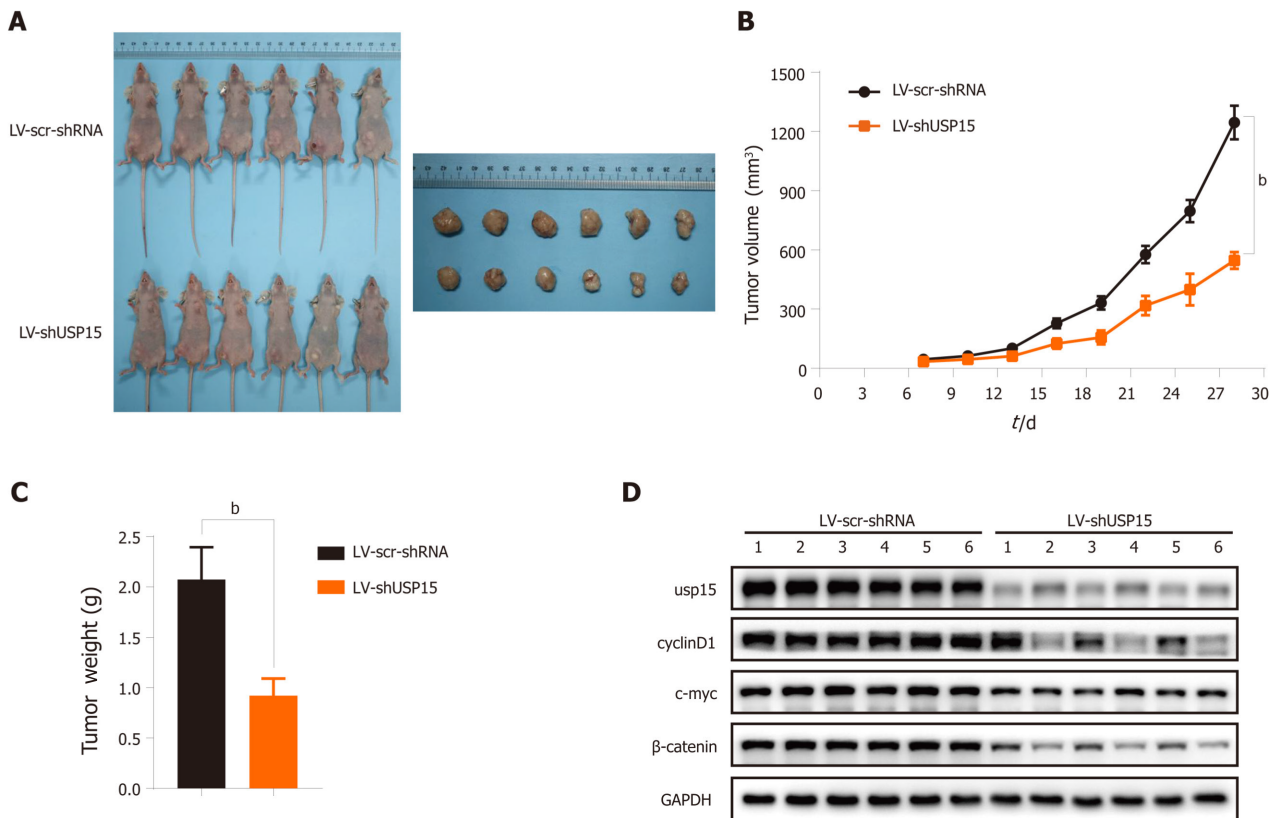


Figure 6 Ubiquitin-specific protease 15 silencing inhibits tumor growth *in vivo*. A: Cervical dislocation was used to sacrifice nude mice on day 29 and representative images of xenograft mouse samples were obtained ($n = 6$); B: Tumor volume was measured every 3 d and a growth curve was drawn; C: Tumor weight was measured when mice were sacrificed; D: Western blotting detected the protein expression of ubiquitin-specific protease 15, β -catenin, c-Myc, and cyclin D1 in tumor samples of two groups. ^b $P < 0.01$, data are expressed as the mean \pm standard error of the mean. USP15: Ubiquitin-specific protease 15.

response[14]. Another recent study showed that USP15 can effectively activate NF- κ B by maintaining the stability of TAB2/3 differentially[38]. In our study, GO enrichment analysis based on RNA-Seq indicated that USP15 regulated the Wnt/ β -catenin signaling pathway in GC. Previous studies have shown that abnormal activation of the Wnt/ β -catenin pathway could promote the malignant progression of a variety of cancers, including GC[39,40]. Increased nuclear expression of β -catenin is an important sign of Wnt/ β -catenin signaling pathway activation, which mainly depends on the transport of cytoplasmic β -catenin to the nucleus[39,40]. In our study, knockdown of USP15 significantly reduced the nuclear expression of β -catenin and downregulation of Wnt/ β -catenin downstream genes in GC cells, while USP15 overexpression yielded opposite results, and there was no change in the USP15 C269S group (USP15 mutant), indicating that USP15 acted as a Wnt/ β -catenin pathway activator. A rescue experiment by using LiCl (a Wnt/ β -catenin pathway activator) showed that the effect of USP15 on GC progression was dependent on Wnt/ β -catenin pathway. All of these findings suggest that USP15 contributes to GC progression by regulating the Wnt/ β -catenin signaling pathway.

To the best of our knowledge, this study is the first to explore the clinical significance and molecular function of USP15 in GC. However, our research had some limitations. This was a retrospective study that included a small number of GC patients from a single center in our hospital, so there may have been a degree of bias. In the future, a large multicenter study should be conducted to verify our results. In addition, although GeneMANIA[41], a protein interaction bioinformatics website, predicts that USP15 can interact with some upstream proteins (CTNNB1, NUSAP1) of the Wnt/ β -catenin pathway, the specific molecular mechanism problems need to be resolved in future research.

CONCLUSION

In conclusion, the results presented in our study demonstrated that USP15 was

upregulated in GC cells and tissues, and was associated with a poor prognosis in patients with GC. Furthermore, USP15 promoted cell proliferation, invasion, and EMT progression *via* the Wnt/ β -catenin signaling pathway *in vitro* and promoted the growth of GC cells *in vivo*. All of our findings shed light on USP15 as a novel promising therapeutic target for understanding the pathogenesis of GC, providing new insights into the development of novel strategies for diagnosis and treatment from the bench to clinic.

ARTICLE HIGHLIGHTS

Research background

Ubiquitin-specific protease 15 (USP15) is an important member of the ubiquitin-specific protease (USP) family, whose expression is dysregulated in many types of cancer. However, the function role and the underlying mechanism of USP15 in gastric cancer (GC) progression have not yet been elucidated.

Research motivation

To explore the underlying mechanisms of GC development and discover biomarkers for the treatment of GC.

Research objectives

To investigate the role and potential mechanism of USP15 in GC.

Research methods

Bioinformatics databases and western blot analysis were utilized to determine the expression of USP15 in GC. Immunohistochemistry was performed to evaluate the correlation between expression of USP15 and clinicopathological characteristics of GC patients. A loss- and gain-of-function experiment was used to investigate the biological effects of USP15 on GC carcinogenesis. RNA sequencing analysis, immunofluorescence, and western blotting were performed to explore the potential mechanism by which USP15 exerted its oncogenic functions.

Research results

USP15 was upregulated in GC tissue and cell lines. The expression level of USP15 was positively correlated with clinical characteristics (tumor size, depth of invasion, lymph node involvement (LNI), tumor-node-metastasis (TNM) stage, perineural invasion, and vascular invasion), and was related to poor prognosis. USP15 knockdown significantly inhibited cell proliferation, invasion and epithelial-mesenchymal transition of GC *in vitro*, while overexpression of USP15 promoted these processes. Knockdown of USP15 inhibited tumor growth *in vivo*. Mechanistically, RNA-seq analysis showed that USP15 regulated the Wnt signaling pathway in GC. Western blotting confirmed that USP15 silencing led to significant downregulation of β -catenin and Wnt/ β -catenin downstream genes (c-myc and cyclin D1), while overexpression of USP15 yielded the opposite results and USP15 mutation showed no change. Immunofluorescence indicated that USP15 promoted the nuclear translocation of β -catenin, suggesting activation of the Wnt/ β -catenin signaling pathway, which may be the critical mechanism promoting GC progression. Finally, rescue experiments showed that the effects of USP15 on gastric cancer progression were dependent on the Wnt/ β -catenin pathway.

Research conclusions

USP15 promotes cell proliferation, invasion, and EMT progression of GC *via* regulating the Wnt/ β -catenin pathway.

Research perspectives

USP15 is expected to be a novel potential therapeutic target for GC.

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