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Basic Study

USP15 contributes to gastric cancer progression *via* regulating the Wnt/β-catenin signaling pathway

Min Zhong, Ling Zhou, Zhi Fang, Yang-Yang Yao, Jian-Ping Zou, Jian-Ping Xiong, Xiao-Jun Xiang, Jun Deng

Abstract

BACKGROUND

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

AIM

To explore the biological role and underlying mechanism 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 expression of USP15 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 analysis, immunofluorescence and western blotting were performed to explore the potential mechanism by which USP15 exerted 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 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: USP15, Gastric cancer, Wnt/β-catenin, Cell proliferation, Cell invasion, EMT.

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Core Tip: Ubiquitin-specific protease 15 (USP15) was up-regulated in gastric cancer (GC) cells and tissues, and was associated with 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 Wnt/β-catenin signaling pathway. USP15 is expected to be a novel potential target for GC therapy.

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 month[3-5]. Therefore, the identification of novel potential targets for GC diagnosis and therapy and elucidation of the underlying mechanism of disease progression are essential for the prevention and treatment of GC.

In recent years, increasing evidences showed that ubiquitin-specific proteases (USPs), the largest deubiquitinase subfamily, playing an important role in GC. For example, USP14[6], USP42[7], and USP44[8] were up-regulated in GC, and could be used as independent prognostic markers in GC patients. USP15, as one of the most important members of the USPs, has been found to have some amplifications in many tumors. The N terminus of the protein encoding USP15 includes a ubiquitin-specific proteases (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 the mutation of Cys269 to Ser (USP15 C269S) can make the enzyme activity disappear[10]. Previous studies have reported that USP15 was up-regulated in liver and pancreatic cancer, and was associated with poor prognosis[11,12]. Mechanistically, USP15 could activate TGF-β signaling pathway and promote progression of advanced malignant glioma by

combining SMURF2 complex and deubiquitinating and thus stabilizing TβR-I[13]. In addition, USP15 could negatively regulate the function of p53 through an effect of de-ubiquitination and stabilization of MDM2. Intriguingly, inhibiting the activity of USP15 can induce tumor apoptosis and improve antitumor T-cell response instead[14]. However, the function of USP15 in GC and its potential mechanism have not been identified.

Wnt/β-catenin signaling pathway is reported to be involved in many cellular processes, such as tumor growth, differentiation and invasion and tumorigenesis[15]. Wnt/β-catenin signaling pathway 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 mechanism of Wnt/β-catenin activation in GC has not been fully elucidated.

We found that USP15 was up-regulated in GC (GC) cells and tissues, and was associated with poor prognosis in GC patients. 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 Wnt/β-catenin signaling pathway. 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 all 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 (DMEM), 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 the methods as described previously[20]. A primary antibody against USP15 (1:100; Cell Signaling Technology, #66310) was used to detect 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 according to the method described previously.

CCK-8 assay and colony formation assay

Forty-eight hours after transfection, 2000 GC cells per well were seeded into a 96-well plate for CCK-8 assay and 1000 GC cells per well were seeded into a six-well plate for colony formation assay following the methods described previously[21].

Wound healing assay

Forty-eight hours after transfection, 5×10^5 GC cells per well were seeded into a six-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. The cells were then carefully

washed with PBS and cultured in serum-free medium. Images were captured at 0, 24 and 48 h to assess wound closure.

Transwell assay

Transwell assay was performed *via* using 8-μm transwell chambers (Merck KGaA, Darmstadt, Germany) with or without 60 μL Matrigel gel (BD Biosciences), 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 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 get the cells attached and grew, and then washed the cells with PBS for three times. At the room temperature, the cells were fixed on a coverslip with 4% tetraformaldehyde for 20 min, and then were washed with PBS for three times again. After 10 mins-incubating with 0.5% Triton X-100, the cells were blocked by incubating 5% bovine serum albumin for 2 h, then anti-β-catenin antibody (1:200 dilution; Cell Signaling Technology) was added into the coverslip overnight at 4°C. After washing three times with PBS, the secondary antibody (1:50 dilution, ab150077, Abcam) was then incubated for 1 h at the room temperature. The coverslips were subsequently washed with phosphate buffer saline (PBS) for three times and then were stained with 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence images of our cells were captured *via* laser confocal microscopy.

Western blotting

Western blotting was performed following the methods described previously[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), GAPDH (1:2000, #60004-1-Ig, Proteintech).

Plasmid construction and cell transfection

To knock down the expression of USP15, three different siRNAs and a negative control were designed as followed: USP15-Homo-249, 5′-GGAACACCUUAUUGAUGAATT-3′; USP15-Homo-1150, 5′-GCAGAUGGAAGGCCAGAUATT-3′; USP15-HoMo-1382, 5′-CCAAACCUAUGCAGUACAATT-3′; and a negative control 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 (Thermo Scientific, R0532, United States).

RNA-sequencing (Seq) analysis

The isolated USP15 knockdown and control BGC-823 cells were used for cDNA amplification and RNA-Seq library preparation. RNA sequencing was performed by Beijing Novel Bioinformatics Co. Ltd. (Beijing, China). Genes with a false discovery rate (FDR) < 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 (sh)RNAs 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 5–6-wk-old BALB/c-nu mice. Tumor volume was measured every 3 d and

calculated according the formula: volume (mm^3) = (length × $width^2$)/2. Mice were killed after 28 d and xenograft tumors were measured and weighed. Proteins were extracted from tumors and USP15 and β-catenin expression were detected by western blotting.

Statistical analysis

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

RESULTS

USP15 is up-regulated in GC cells and tissues, and is associated with poor prognosis in patients with GC

Firstly, 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 was higher than that 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 gastric epithelial cell line GES-1 (Figure 1D). USP15 was elevated in most of eight pairs of clinical GC tissues and their adjacent normal tissues (Figure 1E).

As USP15 was found to be up-regulated 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 that in GC tissues, which was 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 between 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 the results showed above, BGC-823 and MKN-45 cells had a 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 then used to confirm the silencing efficiency of USP15 in GC cells (Figure 2A). The result 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 negative control (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 up-regulated E-cadherin and down-regulated 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 negative control (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 up-regulated vimentin and N-cadherin but down-regulated 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 negative control 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 up-regulated and transcripts of 1209 were down-regulated in USP15 knockdown groups compared to the control groups (Figure 4C). GO enrichment analysis 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 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).

LiCl 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 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 when treating them with LiCl compared with 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). Above findings suggested that the function of USP15 on GC progression was dependent on Wnt/β-catenin pathway.

USP15 knockdown inhibits tumor growth in vivo

We investigated the function of USP15 *in vivo*. BGC-823cells transfected with LV-shUSP15 or LV-scramble shRNA were subcutaneously injected into nude mice to establish a xenograft mouse model. After the mice were killed 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, which was consistent with the *in vitro* results (Figure 6D).

DISCUSSION

In recent years, more and more 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 is related to poor prognosis of breast cancer[28,29]. Two recent studies have shown that USP15 was up-regulated in liver cancer and pancreatic ductal cell carcinoma[11,12]. In this study, immunohistochemical analysis 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 was 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*. Subsequnently, we further identified that USP15 could 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 evidences showed that tumor cells after EMT had high motility and aggressiveness, among which E-cadherin, N-cadherin, and vimentin were important molecular markers[32]. In addition, the epithelial marker E-cadherin was down-regulated, while the mesenchymal markers vimentin and N-cadherin were up-regulated 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 could 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], COP9 signaling body[34], p53 signaling pathway[14], and nuclear factor NF-κB[35]. For example, USP15 promotes the stabilization of TGF-β

receptor and its downstream signal transducers, thereby resulting in an enhance in 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 protein level of p53, and inactivation of USP15 can induce tumor apoptosis and improve antitumor T-cell response[14]. Another recent study showed that USP15 could effectively activate NF-κB 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 these findings suggested that USP15 contributed to GC progression *via* regulating the Wnt/β-catenin signaling pathway.

To our knowledge, this study is the first study 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 up-regulated in GC cells and tissues, and was associated with 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 our findings shed great light on USP15 as a novel promising therapeutic target for deciphering pathogenesis of GC, providing new insights into development of new strategies in diagnosis and treatment from bench to clinic.

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