USP5 promotes tumor progression by stabilizing SLUG in bladder cancer

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Received April 16, 2024; Accepted August 7, 2024

DOI: 10.3892/ol.2024.14705

Abstract. Bladder cancer ranks as the second most prevalent urology malignancy globally. Invasive metastasis is a significant contributor to mortality among patients with bladder cancer, yet the underlying mechanisms remain elusive. Deubiquitinases are pivotal in carcinogenesis, with USP5 implicated in the malignant progression of hepatocellular carcinoma, colorectal cancer and non-small cell lung cancer. The present study assessed the role and mechanism of ubiquitin-specific proteinase 5 (USP5) in the malignant progression of bladder cancer. The association between USP5 expression and bladder cancer prognosis and stage was analyzed using The Cancer Genome Atlas database. Moreover, to elucidate the role of USP5 in bladder cancer, USP5 overexpression and knockdown cell lines were established using T24 cells. Cell viability, proliferation and migration were assessed using Cell Counting Kit-8, Transwell and scratch assays, respectively. Cyclohexanamide was used to evaluate the effect of USP5 expression on Snail family zinc finger 2 (SLUG) stability. Immunoprecipitation and immunofluorescence co-localization were utilized to probe the interaction between USP5 and SLUG. Changes in mRNA and protein levels were assessed using reverse transcription-quantitative PCR and western blotting, respectively. The results revealed that patients with bladder cancer with high USP5 expression had significantly shorter survival (P<0.05) and a higher clinicopathologic stage (P<0.05) than those with low USP5 expression. T24 cells overexpressing USP5 demonstrated significantly increased proliferation (P<0.05), invasion (P<0.05) and expression of epithelial-mesenchymal transition markers (P<0.05); whereas T24 cells with knocked-down USP5 expression revealed significantly reduced proliferation (P<0.05), invasion (P<0.05) and epithelial-mesenchymal transition markers (P<0.05). Immunoprecipitation experiments demonstrated the binding of USP5 to SLUG in bladder cancer cells, with further analysis revealing that USP5 upregulated protein levels of SLUG by inhibiting its ubiquitination. Furthermore, the treatment of bladder cancer cells with Degrasyn, a USP5 inhibitor, was associated with a significant inhibition of the proliferation (P<0.05) and invasion (P<0.05) of T24 cells. In conclusion, the findings of the present study underscore the role of USP5 in promoting the malignant progression of bladder cancer through the stabilization of SLUG. Targeting USP5 holds promise as a strategy for inhibiting bladder cancer progression.

Introduction

Bladder cancer is the second most prevalent malignant tumor of the urological system (5.85 cases per 100 000), and has inflicted a considerable human and economic toll worldwide (1). Over recent decades, concerted efforts in cancer prevention and treatment have led to a significant decline in bladder cancer mortality rates (2.37 cases per 100 000) (2). Nevertheless, patients with bladder cancer often face the challenge of tumor cell metastasis, and the prognosis for patients with metastatic bladder cancer remains unfavorable (3). Therefore, it is imperative to elucidate the underlying mechanisms of bladder cancer cell infiltration and metastasis, as this knowledge is pivotal for devising effective treatment strategies against bladder cancer.

Deubiquitinating enzymes (DUBs) are proteases that catalyze the hydrolysis of ubiquitin moieties from ubiquitinated substrates or polyubiquitin chains, serving pivotal roles in maintaining cellular homeostasis of protein quantity and activity (4). Numerous studies have highlighted the involvement of DUBs in regulating crucial aspects of cancer biology such as cell proliferation, invasion, metastasis, DNA damage repair, metabolism and drug sensitivity (5-9). Ubiquitin-specific proteinase (USP)5 is a cysteine deubiquitinating enzyme belonging to the USP family, which engages in diverse physiopathological processes in several cancer types (10-12). In hepatocellular carcinoma, USP5 has been reported to promote epithelial-mesenchymal transition (EMT) by stabilizing Snail family zinc finger 2 (SLUG) (13). Similarly, in breast cancer, USP5 has been reported to facilitate cancer cell proliferation

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Key words: ubiquitin-specific proteinase 5, bladder cancer, Snail family zinc finger 2, epithelial-mesenchymal transition

and metastasis by stabilizing hypoxia-inducible factor-2α (14). Moreover, in non-small cell lung cancer, USP5 acts as a deubiquitinase, promoting cancer cell proliferation through the stabilization of cyclin D1 (15).

There is mounting evidence emphasizing the pivotal role of EMT in tumor progression and metastasis (16). EMT entails a predominant decrease in the expression of epithelial cell markers, such as E-cadherin, alongside an increase in the expression of mesenchymal cell markers, including vimentin (VIM) and N-cadherin. This process augments tumor progression by enhancing tumor cell stemness and diminishing tumor cell apoptosis. EMT has been identified as a critical process endowing tumor cells with the ability to metastasize and invade (17-19). In bladder cancer, EMT is intimately associated with malignant transformation, contributing significantly to tumor progression and invasion (20). Despite its recognized significance as a predictor of bladder cancer metastasis and its association with drug resistance, the precise underlying mechanisms of EMT in bladder cancer remain incompletely understood (21,22).

The aim of the present study was to elucidate the role and mechanism of USP5 in the progression of bladder cancer. An analysis of the relationship between USP5 expression and the clinicopathological stage and prognosis of bladder cancer was performed using clinical databases. Furthermore, employing immunoprecipitation and other experimental techniques, the present study assessed how USP5 facilitates the proliferation, invasion and EMT process of bladder cancer cells by stabilizing SLUG.

Materials and methods

Cell culture and drugs. The human bladder urothelial carcinoma T24 cell line was purchased from Procell Life Science & Technology Co., Ltd. These cells were cultured in a 5% CO_2 atmosphere at 37˚C, utilizing 5A medium supplemented with 10% fetal bovine serum (both Pricella Biotechnology Co., Ltd.) and 1% penicillin-streptomycin. The USP5 inhibitor, Degrasyn, was purchased from MedChemExpress. Based on the Cell-Counting Kit-8 (CCK-8) experiment, it was demonstrated that the half maximal inhibitory concentration (IC_{50}) of Degrasyn for T24 cells was 2.04 μg/ml and this was determined to be the working concentration in the subsequent experiments.

Bioinformatics analysis based on The Cancer Genome Atlas (TCGA) database. Bulk RNA sequencing data from patients with bladder cancer were sourced from TCGA-BLCA (portal.gdc.cancer.gov). Patients within the TCGA dataset were stratified into two groups based on USP5 expression levels: Low- and high-expression. Survival probabilities for overall survival were estimated using the Kaplan-Meier method. Bioinformatics analysis and visualization of differential USP5 expression across several clinicopathological stages were performed using R.4.1 software (The R Foundation). Correlation analysis of USP5 with EMT-related genes was performed using Gene Expression Profiling Interactive Analysis 2.0 (gepia2.cancer-pku.cn). GeneMANIA database (genemania.org) was used to analyze the protein interaction network of USP5.

Tissue samples and immunohistochemical (IHC) assay. All clinical samples for IHC were obtained from the Department of Urology, the First Affiliated Hospital of Bengbu Medical University (Bengbu, China) and written informed consent was obtained from the patients. Specimens were obtained from patients with clinically confirmed bladder cancer, aged 58-76 years, with a median age of 62 years (16 male and 9 females). The inclusion criteria were as follows: i) Diagnosis with bladder cancer using a pathological method; ii) No treatment other than surgery, such as immunotherapy and chemotherapy; iii) age ≥18 years old; The exclusion criteria were as follows: i) Additional malignant diseases; ii) absence of measurable lesion to be assessed; iii) refusal to provide tissue for research. All patients were pathologically diagnosed with bladder cancer from July 2022 to May 2023. The use of clinical samples in this study was approved by the First Affiliated Hospital of Bengbu Medical University and the study protocols was performed in accordance with ethical principles and local legislation.

The tissue samples were fixed using 4% paraformaldehyde at room temperature for 2 h. All paraffin-embedded specimens were cut into 4-μm-thick sections and mounted on glass slides. After dewaxing with xylene and rehydrated in decreasing concentration of ethanol, antigen retrieval was performed in a microwave (100˚C for 7 min). Endogenous peroxidase activity was quenched with 0.3% H_2O_2 for 30 min at room temperature. Non-specific binding sites were blocked by incubation with Immunol Staining Blocking Buffer (Beyotime, P P0102) for 1 h at room temperature. Subsequently, the samples were incubated with USP5 antibody (Proteintech Group, Inc.; cat. no. 10473-1, 1:100) at 4˚C overnight, followed by incubation with secondary biotinylated antibodies (Proteintech Group, SA00004-2, 1:500) for 30 min at 37˚C. Visualization was achieved using a DAB solution, and counterstaining was performed with hematoxylin at room temperature for 1 min. Photomicrographs were captured using an optical microscope.

Transfection. USP5 and SLUG overexpression (PCDNA3.1) and knockdown (pLKO.1) plasmids were purchased from Wuhan Genecreate Biological Engineering Co., Ltd. The target sequence of short hairpin shUSP5 was 5'-CTTTGCCTTCAT TAGTCACAT-3'; the target sequence of shSLUG was 5'-CAG CTGTAAATACTGTGACAA-3'; and the target sequence of the scrambled shRNA control was 5'-CCTAAGGTTAAG TCGCCCTCG-3'. T24 cells were seeded in six-well plates ($5x10^5$/well) and transfected with the plasmids (1 μg/well) using Lipofectamine 3000 Transfection Reagent (L3000015, Thermo Fisher Scientific, Inc.). The transfection duration was 12 h at 37˚C, after which the medium was replaced. After continuing the incubation for 48 h, the cells were collected for subsequent experiments. The transfected cells underwent screening with puromycin and were subsequently validated for transfection efficiency through western blotting and PCR.

Western blot. T24 cells were lysed using RIPA buffer (Beyotime, P0013), and protein concentration was measured using a BCA protein determination kit. A total of 30 μg proteins were loaded onto SDS-PAGE gels (10%). After electrophoresis, proteins were electrotransferred to a polyvinylidene fluoride membrane, which was blocked with 5% BSA

(cat. no. PS108P; Epizyme Biotechnology Co., Ltd.) at room temperature for 30 min. The membranes were then incubated with USP5 primary antibodies (1:1,000; cat. no. P45974; Abmart Pharmaceutical Technology Co., Ltd.), SLUG (1:1,000; cat. no. O95863; Abmart Pharmaceutical Technology Co., Ltd.), E-cadherin (1:1,000; cat. no. P12830; Abmart Pharmaceutical Technology Co., Ltd.), N-cadherin (1:1,000; cat. no. P19022; Abmart Pharmaceutical Technology Co., Ltd.), VIM (1:1,000; cat. no. P08670; Abmart Pharmaceutical Technology Co., Ltd.) or GAPDH (1:5,000; cat. no. M20006; Abmart Pharmaceutical Technology Co., Ltd.) at 4˚C overnight, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (1:5,000; cat. no. M21003; Abmart Pharmaceutical Technology Co., Ltd.) for 1 h at room temperature. Membranes were incubated for 3 min in Femto Light Chemiluminescence Kit (cat. no. SQ201; Epizyme Biotechnology Co., Ltd.) and images were captured using the ChemiDoc™ Imaging System(Bio-Rad Laboratories, Inc.).

Reverse-transcription (RT)-quantitative (q)PCR. The cells were lysed using TRIzol (cat. no. 15596018CN; Invitrogen; Thermo Fisher Scientific, Inc.), and complementary DNA was synthesized using a SweScript RT II First Strand cDNA Synthesis Kit (Servicebio, G3332) at 25˚C for 5 min, 55˚C for 10 min and 85˚C for 5 sec. qPCR assays were performed following the manufacturer's instructions using SweScript One-Step RT-PCR Kit (Servicebio, G3335). PCR conditions were one cycle of 50˚C for 15 min; one cycle of 98˚C for 2 min; forty cycles of 98˚C for 15 sec, 60˚C for 20 sec and 72˚C for 10 sec; and one cycle of 72˚C for 5 min) Primers used in the assays were purchased from Wuhan Genecreate Biological Engineering Co., Ltd. Relative expression levels were assessed using the $2^{-\Delta\Delta Cq}$ method (23). The primer sequences employed were as follows: USP5 primers: Forward, 5'-CCACGAACA ATAGTTTAGAACG-3' and reverse, 5'-AGGTCCCACTGG CACAGA-3'; SLUG primers: Forward, 5'-CTTCCTGGTCAA GAAGCA-3' and reverse, 5'-GGGAAATAATCACTGTAT GTGTG-3'; and GAPDH primers: Forward, 5'-GCACCGTCA AGGCTGAGAAC-3' and reverse, 5'-TGGTGAAGACGC CAGTGGA-3'.

Scratch, cell invasion and migration assays. T24 cells were cultured in six-well plates ($5x10^5$/well) until they reached 100% confluence, after which a wound was scratched in the cell monolayer. To mitigate the influence of cell proliferation, T24 cells were cultured in serum-free medium. Images of the wound area were captured at 0 and 24 h. A total of three random views were selected and the number of migrated cells were counted (Image J 1.8.0; National Institutes of Health) using a light microscope (Olympus).

The invasion ability of T24 cells was assessed using a 24-well Transwell chamber equipped with polycarbonate membranes coated with Matrigel. Cells ($1x10^5$) suspended in fresh serum-free 5A medium were seeded into the upper chamber of Transwell plates (8-μm pore size; Costar; Corning, Inc.) precoated with Matrigel at 37˚C for 30 min, and 5A' medium containing 10% FBS was added to the lower chamber. After a 24-h incubation at 37˚C, cells that passed through the Matrigel were stained with 0.5% crystal violet at room temperature for 10 min and were captured under a light microscope (Olympus). The results were analyzed using ImageJ software (version 1.8.0).

CCK-8 assay. Cell survival rates were determined using the CCK-8 assay. A total of ~$5x10^3$ cells were seeded into each well of a 96-well plate, with 100 μl medium per well. Following cell incubation, 10 μl CCK-8 solution (Servicebio; cat. no. G4103) was added to each well and incubated for 2 h away from light. The absorbance was then measured at 450 nm to assess cell viability.

Immunofluorescence co-localization. The samples were fixed using 4% paraformaldehyde at room temperature for 2 h, embedded in paraffin and sectioned to 4-μm thickness. The tissue slides were deparaffinized, hydrated and subjected to heat-mediated antigen retrieval as aforementioned. Following this, goat serum (Servicebio, G1208) was applied to the sections as a blocking agent for 30 min at room temperature. Subsequently, the sections were incubated overnight at 4˚C with primary antibodies against USP5 (1:100; cat. no. P45974; Abmart Pharmaceutical Technology Co., Ltd.) and SLUG (1:100; cat. no. O95863; Abmart Pharmaceutical Technology Co.,Ltd.), followed by incubation with anti-mouseAF594-conjugated (1:100; cat. no. M213627; Abmart Pharmaceutical Technology Co., Ltd.) or -rabbit conjugated FITC (1:100; cat. no. M212315; Abmart Pharmaceutical Technology Co., Ltd.) secondary antibodies (1:5,000; cat. no. M21003; Abmart Pharmaceutical Technology Co., Ltd.) at 37˚C for 2 h. Nuclei were counterstained with DAPI (37˚C, 10 min), and images were captured using a confocal microscope. For immunofluorescent colocalization analysis, the Colocalization Finder and Scatter J plugins of Image J 1.8.0 software (National Institutes of Health) were used. The criteria for colocalization were set as follows: Pearson correlation coefficient (r) >0.5 and Manders' colocalization coefficients (M1 and M2) >0.5.

Protein docking. The protein structures of USP5 and SLUG were obtained from the Protein Data Bank (rcsb.org/). Molecular docking models of the USP5-SLUG interaction were generated using Global RAnge Molecular Matching 1.0 (vakser.compbio.ku.edu/resources/gramm/grammx). The docking poses were assessed based on their scores, and the pose with the highest score was selected as the final conformation.

Coimmunoprecipitation assay. For exogenous immunoprecipitation, co-transfected cells expressing Flag-tagged USP5 and HA-tagged SLUG were generated as aforementioned. The experiment was carried out by Flag IP/Co-IP Kit (Epizyme Biotech, YJ208). Lysis buffer (Epizyme Biotech) was added (30 μl per $1.0x10^5$ cells, along with protease inhibitor (Epizyme Biotech, GRF101). After 20 min of incubation on ice, the cell lysate mixture was centrifuged to collect the supernatant (4˚C, 12,000 g, 10 min). The cell lysates (500 μl) were incubated with Anti-Flag beads (25 μl) at 4˚C for 4 h. Magnetic beads were collected using a magnetic rack and subsequently washed three times using rinse buffer (Epizyme Biotech). Following denaturing elution (boiling), the supernatant was subjected to western blot analysis as aforementioned using the specified antibodies. Diluted whole cell lysate as control.

For endogenous immunoprecipitation, an immunoprecipitation assay was performed using USP5 antibodies (5 μg/mg; cat. no. ab241311; Abcam) and Classic Magnetic Protein A/G IP/Co-IP Kit (YJ201, Epizyme Biotech). Cell lysis steps as before. The beads were mixed with USP5 antibodies at a final concentration of 5 μg/ml and incubated for 2 h. Subsequently, cell lysates were combined with the antibody-magnetic-bead complexes and incubated at 4˚C overnight. Magnetic bead separation, washing and elution steps as before. After elution, the resulting supernatant was subjected to western blot analysis.

Statistical analysis. Differences were assessed via Pearson's correlation test, the log-rank test, Wilcoxon rank-sum test, the unpaired Student's t-test for differences between two groups, and one-way ANOVA, followed by the Bonferroni test for differences between three groups. Analyses were performed using GraphPad Prism version 9.20 (Dotmatics), R version 4.1.0 (The R Foundation) and SPSS 25.0 (IBM Corp.) software. $P<0.05$ was considered to indicate a statistically significant difference. All experiments were performed independently ≥3 times.

Results

USP5 is positively associated with tumor progression in patients with bladder cancer. To assess the expression of USP5 in patients with bladder cancer, data from TCGA-BLCA dataset and specimens from the First Affiliated Hospital of Bengbu Medical University were analyzed. First, a prognostic analysis of USP5 expression in 406 patients with urothelial carcinoma of the bladder was performed using data from TCGA database. The results demonstrated that high USP5 expression was associated with worse overall survival, in comparison with low expression of USP5 ($P<0.05$; Fig. 1A). Subsequently, the differences in USP5 expression in patients with bladder cancer with different tumor (T)-stages and node (N)-stages in the TCGA-BLCA database were analyzed, and the results revealed that USP5 expression was significantly associated with higher T-stages ($P<0.05$; Fig. 1B) and higher N-stages ($P<0.05$; Fig. 1C). cadherin 2 (CDH2), acetyl-CoA acetyltransferase 2 (ACAT2) and VIM, as important markers for EMT, are strongly associated with bladder cancer progression (17-19), and the mRNA expression of f USP5 was significantly positively correlated with CDH2, ACAT2 and VIM, respectively ($P<0.05$; Fig. 1D). Finally, using specimens from the First Affiliated Hospital of Bengbu Medical University, it was demonstrated that USP5 expression was markedly higher in non-muscle invasive bladder cancer than in para-cancerous control tissues, and in muscle invasive bladder cancer tissues than in non-muscle invasive bladder cancer (Fig. 1E). Overall, the aforementioned results indicate that USP5 is significantly and strongly associated with EMT, prognosis and a higher stage in bladder cancer.

USP5 promotes the malignant phenotype of T24 human bladder cancer cells. To assess the biological role of USP5 in bladder cancer cells, the human bladder uroepithelial carcinoma T24 cell line was chosen as a study object. It was demonstrated that USP5 overexpression and knockdown T24 cell lines were constructed using plasmids and the transfection efficiency was assessed using western blotting (Fig. 2A). Scratch and Transwell assays were used to analyze the effect of USP5 on migration and invasion of the bladder cancer T24 cell line. The scratch assays results revealed significantly increased migration in the USP5 overexpressing cell line and significantly decreased migration in the USP5 knockdown cell line compared with the control ($P<0.05$; Fig. 2B). Furthermore, there was a significant increase in the number of invaded cells in the USP5 overexpressing cell line and a significant decrease in the number of invaded cells in the USP5 knockdown cell line compared with the control group in the Transwell assays ($P<0.05$; Fig. 2C). Regarding the proliferative capacity of the cells, the CCK-8 experiments demonstrated that USP5 overexpressing T24 cells had significantly increased proliferation and USP5 knockdown T24 cells had significantly decreased proliferation compared with the control group ($P<0.05$; Fig. 2D). Considering the bioinformatics analyses in this study showing that USP5 is strongly associated with EMT in bladder cancer, western blotting was used to analyze changes in EMT markers (E-cadherin, N-cadherin and VIM) in USP5 overexpressing and knockdown cell lines. The results revealed that E-cadherin expression was significantly decreased, and N-cadherin and VIM expression was significantly increased in the USP5 overexpressing cell line, and the opposite was demonstrated for the USP5 knockdown cell line compared with the control group ($P<0.05$; Fig. 2E). These results indicate that USP5 significantly promotes the malignant phenotype of T24 bladder cancer cells.

USP5 interacts with SLUG in human bladder cancer T24 cells. Certain studies have reported that USP5 promotes EMT by stabilizing SLUG in hepatocellular carcinoma (12,13). Using the GeneMANIA database, the protein interactions network of USP5 were analyzed. The results demonstrated that SLUG is an important interacting protein of USP5 (Fig. 3A). Furthermore, to evaluate the relationship between USP5 and SLUG in the T24 cell line, immunofluorescence co-localization and immunoprecipitation assays were performed. The results revealed that in T24 cells, the fluorescence of USP5 and SLUG was highly overlapping in the nucleus, implying that USP5 and SLUG interact with each other in the nucleus ($r=0.874$; Fig. 3B). The protein structures of USP5 and SLUG also demonstrated the most likely binding forms of USP5 and SLUG in molecular docking analyses (Fig. 3C). Moreover, endogenous and exogenous immunoprecipitation revealed that USP5 and SLUG interacted in human bladder cancer T24 cells (Fig. 3D). Overall, the aforementioned experimental results indicate that SLUG is an important reciprocal protein of USP5 in bladder cancer cells.

USP5 stabilizes SLUG by reducing its ubiquitination. To assess the effect of USP5 expression on the protein level of SLUG, the expression level of SLUG in T24 cell lines with USP5 overexpression or knockdown was analyzed using western blotting. The results revealed that the protein level of SLUG was significantly increased in the USP5 overexpression T24 cells and significantly decreased in the USP5 knockdown T24 cells compared with the control ($P<0.05$; Fig. 4A). Conversely, RT-qPCR demonstrated that USP5 overexpression or knockdown did not significantly affect the RNA transcript levels of SLUG ($P>0.05$; Fig. 4B). These results suggest that USP5 is

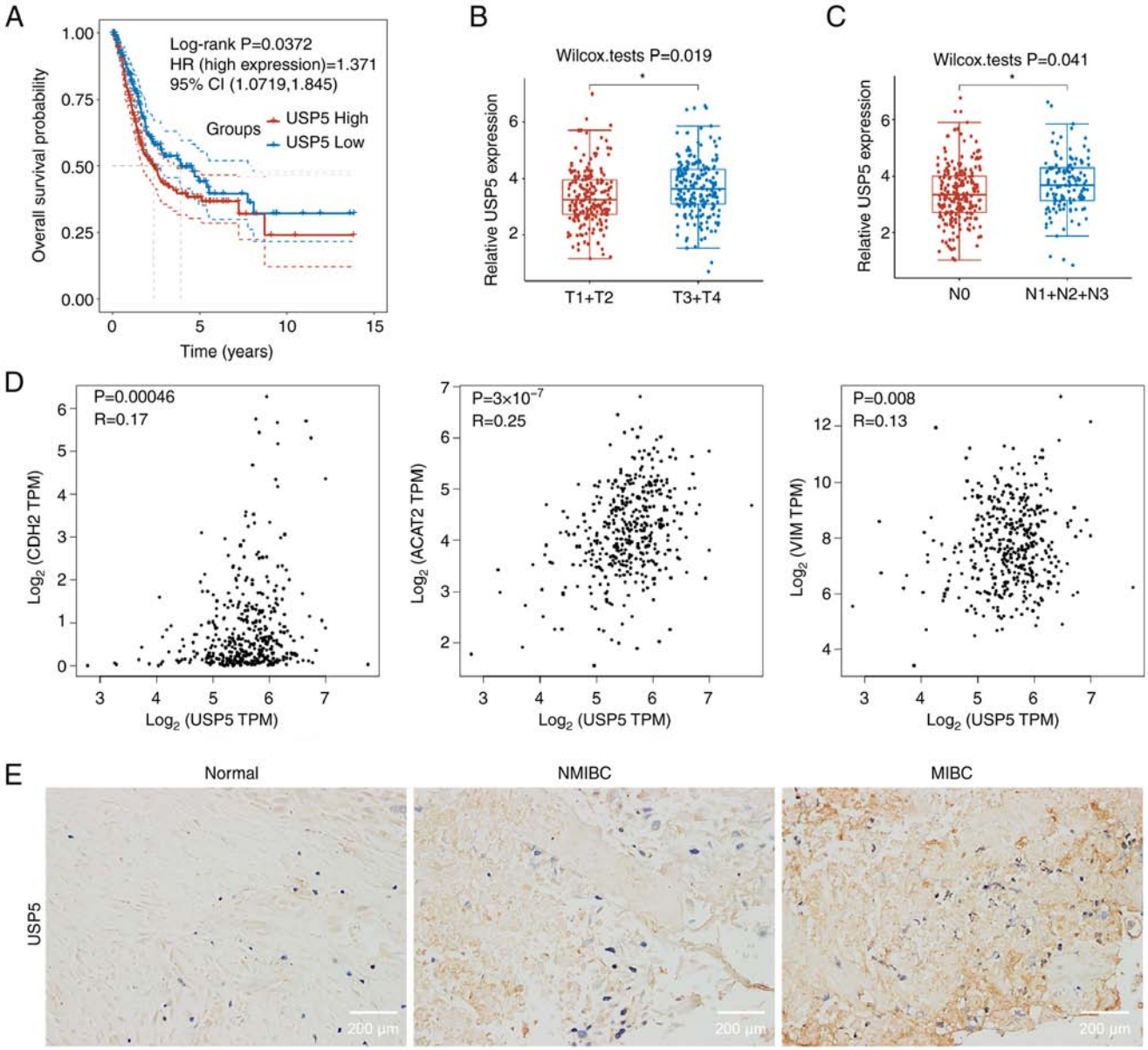

Figure 1. USP5 expression is associated with tumor progression in patients with bladder cancer. (A) Relationship between the USP5 expression level and overall survival using data from TCGA-BLCA dataset. Analysis of USP5 expression in different (B) T and (C) N stages using data from TCGA-BLCA dataset. (D) Correlation analysis of USP5 with epithelial-mesenchymal transition markers (CDH2, ACAT2 and VIM) using data from TCGA-BLCA dataset. (E) Representative immunohistochemistry staining images of USP5 in normal bladder tissue, NMIBC and MIBC. *P<0.05. USP5, ubiquitin-specific proteinase 5; TCGA-BLCA, The Cancer Genome Atlas-Bladder cancer; T, tumor; N, node; CDH2, cadherin 2; ACAT2, acetyl-CoA acetyltransferase 2; VIM, vimentin; NMIBC, non-basal invasive bladder cancer; MICB, muscle invasive bladder cancer; TPM, transcripts per million; HR, hazard ratio; CI, confidence interval.

likely to regulate SLUG RNA levels through post-translational modifications. Therefore, to assess whether USP5 expression affected the post-translational stability of SLUG, the changes in protein levels of SLUG at different times in USP5 overexpressing or knockdown cells were compared after the addition of the protein synthesis inhibitor cyclohexanamide. The results demonstrated that SLUG degradation was significantly slowed down in USP5 overexpressing cells and significantly accelerated in USP5 knockdown cells (P<0.05; Fig. 4C). Furthermore, as USP5 is an important deubiquitinating enzyme, the deubiquitination of SLUG by USP5 in T24 cells was explored. After immunoprecipitation using SLUG antibodies, the expression level of ubiquitin was detected using western blotting under the condition that the same amount of SLUG was present in the immunoprecipitated product. The results revealed that ubiquitination levels of SLUG were markedly reduced in USP5 overexpressing cells and increased in USP5 knockdown cells (Fig. 4D). These results indicate that USP5 regulates SLUG protein levels post-translationally, mainly through deubiquitination.

USP5 promote the progression of bladder cancer by interacting with SLUG. SLUG expression was knocked down in USP5-overexpressing cells to assess whether USP5 enhances the malignant phenotype of bladder cancer cells primarily through SLUG. Compared with the vector group, USP5

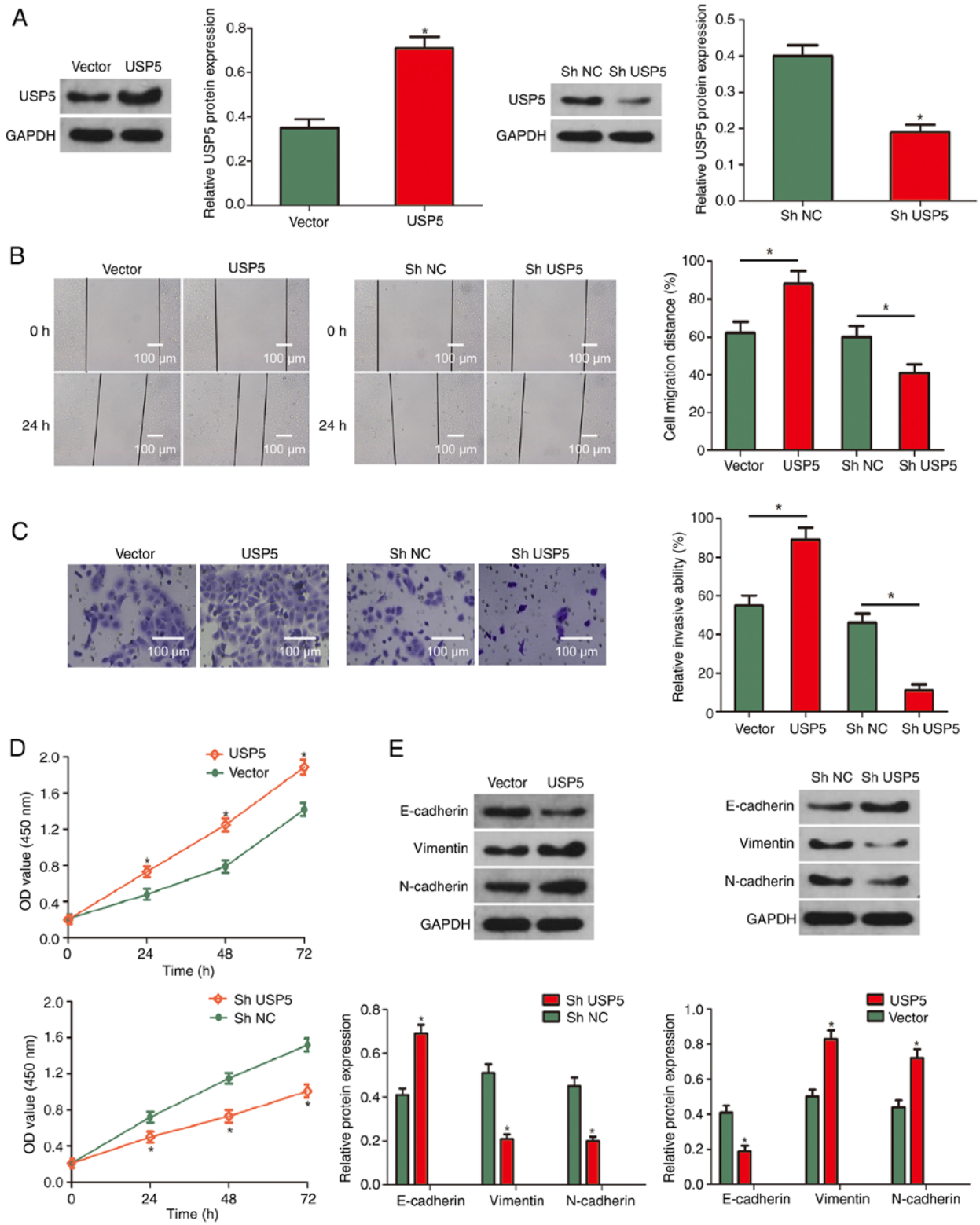

Figure 2. USP5 promotes the malignant phenotype of human bladder cancer T24 cells. (A) Protein expression levels of USP5 in knockdown and overexpression human bladder cancer T24 cell lines. (B) Cell scratch test results of human bladder cancer T24 cells after knockdown and overexpression of USP5. Images were captured with a light microscope at 0 and 24 h. Results of the (C) Transwell and (D) cell proliferation assays after the knockdown and overexpression of USP5. (E) Western blot results demonstrate the expression of epithelial-mesenchymal transition-related proteins (E-cadherin, N-cadherin and vimentin) in human bladder cancer T24 cells after the knockdown and overexpression of USP5. *P<0.05. USP5, ubiquitin-specific proteinase 5; sh, short hairpin; NC, negative control; OD, optical density.

was successfully overexpressed in the USP5 group, and the level of SLUG was successfully knocked down in the USP5 + shSLUG group compared to that in the USP group (P<0.01; Fig. 5A and B). Knockdown of SLUG in USP5 + shSLUG group significantly inhibited the tumor cell invasiveness compared to the USP group in the Transwell assay

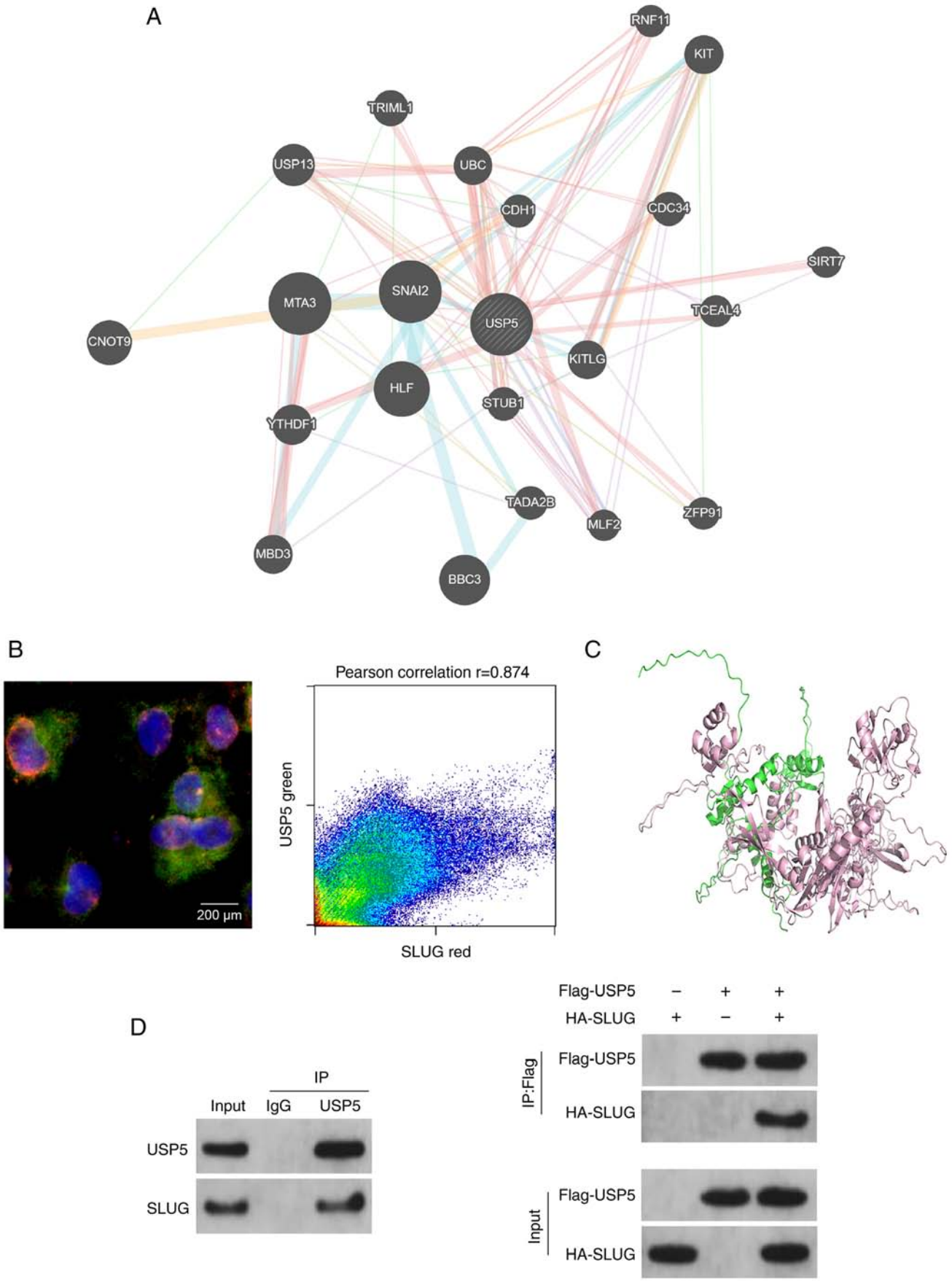

Figure 3. USP5 interacts with SLUG in human bladder cancer T24 cells. (A) Protein interaction network map of proteins bound to USP5 using data from the GeneMANIA database. (B) Colocalization of USP5 and SLUG in T24 cells was visualized using a confocal microscope, and ImageJ software was used for colocalization analysis of USP5 and SLUG expression. Pearson correlation analysis revealed that r>0.5, and Manders' colocalization coefficient demonstrated that M1>0.5 and M2 >0.5. (C) Computational docking model of USP5 and SLUG. (D) Endogenous and exogenous IP demonstrated the interaction of USP5 and SLUG in T24 cells. USP5, ubiquitin-specific proteinase 5; SLUG, Snail family zinc finger 2; IP, immunoprecipitation.

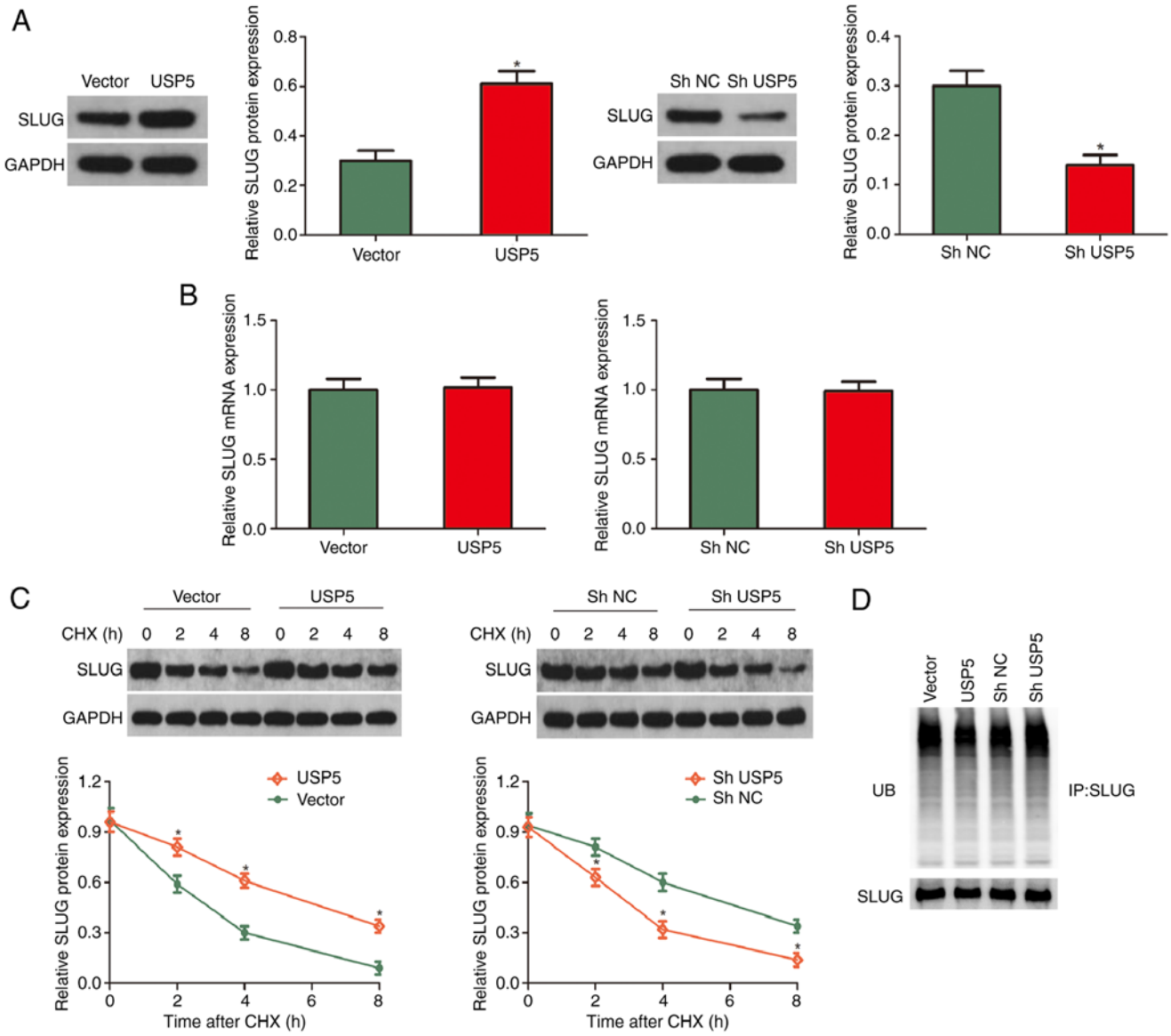

Figure 4. USP5 stabilizes SLUG by reducing its ubiquitination. (A) Protein expression and (B) mRNA levels of SLUG in the USP5 knockdown and overexpression T24 cell lines. (C) Remaining SLUG protein levels in the USP5 knockdown and overexpression T24 cell lines after treatment with CHX at several time points. (D) Western blotting revealed that USP5 regulated SLUG ubiquitination in T24 cells. SLUG proteins were isolated from the USP5 knockdown or overexpression T24 cells by Co-IP, and the ubiquitination of vimentin was then detected using western blotting. *P<0.05. USP5, ubiquitin-specific proteinase 5; SLUG, Snail family zinc finger 2; IP, immunoprecipitation; CHX, cyclohexanamide; sh, short hairpin; NC, negative control; UB, ubiquitination.

(P<0.01; Fig. 5C). Moreover, SLUG knockdown in USP5 + shSLUG group significantly alleviated the cell proliferation compared to the USP group in the CCK-8 analysis (P<0.01; Fig. 5D). Additionally, SLUG knockdown in USP5 + shSLUG group significantly alleviated the tumor EMT compared to the USP group (P<0.01; Fig. 5E). The aforementioned experimental results indicate that, in bladder cancer cells, USP5 enhances the proliferation, invasion and EMT process of tumor cells, mainly through SLUG.

Pharmacological inhibition of USP5 suppresses bladder cancer progression. Several drugs have been developed to target USP5, among which Degrasyn was chosen to assess its inhibitory effect on bladder cancer. First, the IC_{50} of Degrasyn was evaluated in the human bladder cancer T24 cell line. The results revealed that the IC_{50} of Degrasyn for the T24 cells was 5.317 μM/ml (2.04 μg/ml; Fig. 6A), and thus 2 μg/ml was determined as the working concentration for the subsequent experiments. Western blotting demonstrated that Degrasyn significantly inhibited the protein expression level of USP5 in USP5 + Degrasyn group compared with that in the USP group (Fig. 6B). Furthermore, RT-qPCR results revealed that Degrasyn had no significant effect on the mRNA levels of USP5 in USP5 + Degrasyn group compared to that in USP group (Fig. 6C). However, Degrasyn in USP5 + Degrasyn group significantly inhibited tumor cell invasiveness compared to the USP group in the Transwell assay (P<0.01; Fig. 6D). Degrasyn in USP5 + Degrasyn group also significantly alleviated cell proliferation compared to the USP group in the CCK-8 analysis (P<0.01; Fig. 6E). In addition, Degrasyn in USP5 + Degrasyn group significantly inhibited the N-cadherin and VIM expression, and

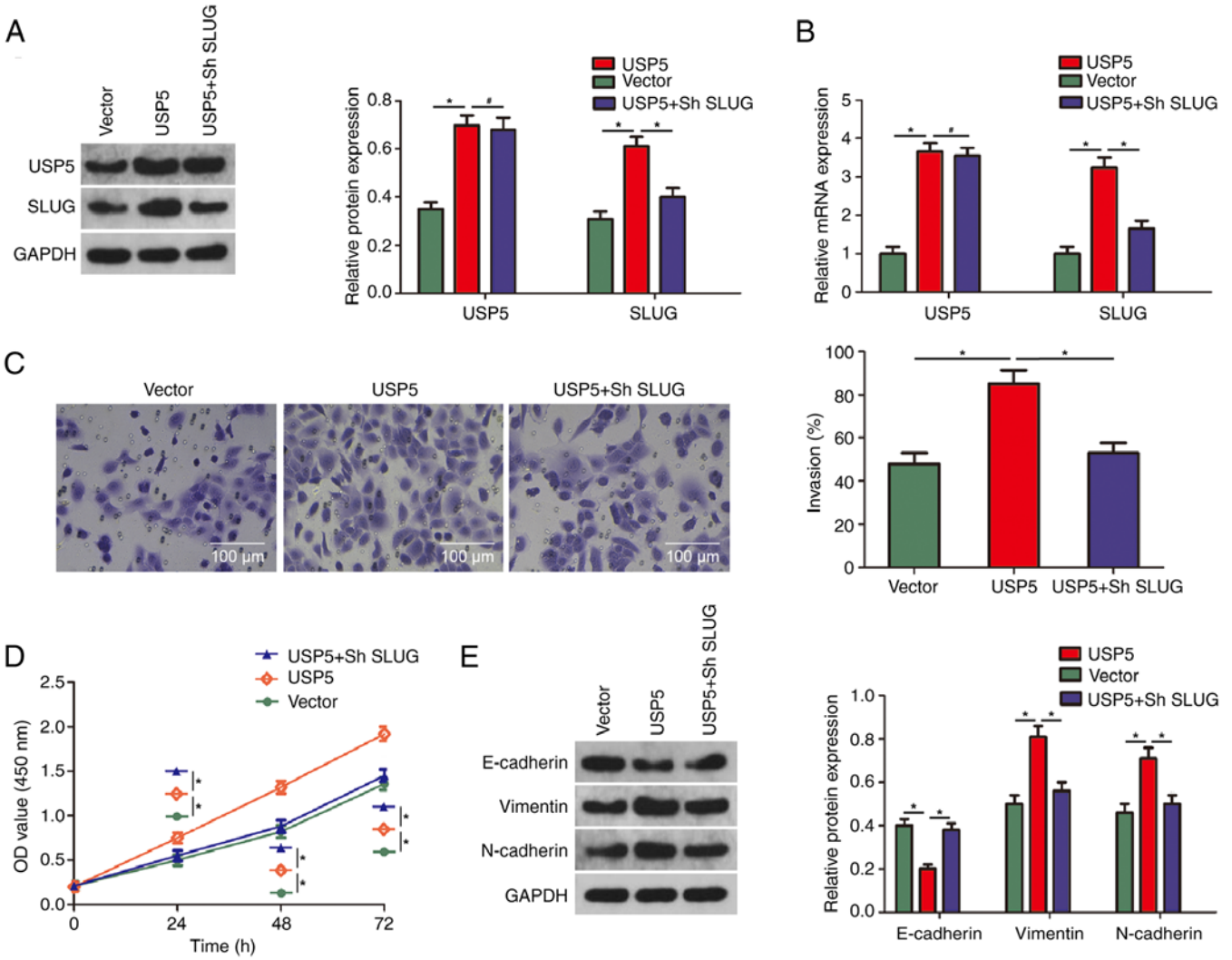

Figure 5. USP5 promotes the progression of bladder cancer by interacting with SLUG. (A) Protein expression and (B) mRNA levels of SLUG in the USP5 overexpression T24 cell lines with and without SLUG knockdown. Results of the (C) Transwell and (D) cell proliferation assays in the USP5 overexpression T24 cell line with or without SLUG knockdown. (E) Protein expression levels of epithelial-mesenchymal transition marker (E-cadherin, N-cadherin and vimentin) in the USP5 overexpression T24 cell lines with or without SLUG knockdown. *P<0.05; #P>0.05. USP5, ubiquitin-specific proteinase 5; SLUG, Snail family zinc finger 2; sh, short hairpin; OD, optical density.

significantly increased the E-cadherin expression compared to the USP group (P<0.01; Fig. 6F). These results suggest that pharmacological inhibition of USP5 significantly suppresses the proliferation, invasion and EMT process of bladder cancer cells.

Discussion

DUBs are indispensable for maintaining ubiquitin homeostasis and are involved in several cellular functions (11). With >100 DUBs encoded in the human genome, USP5, also known as ubiquitin isopeptidase, has been implicated in diverse cellular processes such as stress responses, DNA repair and inflammatory responses (24,25). Moreover, USP5 has been associated with several cancers including breast, pancreatic, hepatic-colonic, prostate and lung (14,25-28). Nonetheless, the specific mechanism of action of USP5 in bladder cancer remains elusive. To address this gap, the present study initially analyzed USP5 expression in bladder uroepithelial carcinoma using data from the TCGA database and clinical specimens. The findings revealed a strong association between USP5 expression and tumor progression, as well as poor prognosis in patients with bladder uroepithelial carcinoma. Consistent with previous reports (14,26-29), the present study further demonstrated in USP5-overexpressing and USP5-knockdown cancer cell lines that USP5 exacerbates the malignant phenotype of bladder cancer cells. Combining these results with the established biological functions of USP5, the present study demonstrated that USP5 promotes the malignant progression of bladder cancer by binding and stabilizing SLUG, thus elucidating one of the key mechanisms underlying USP5-mediated bladder cancer progression.

Modifications, including phosphorylation, ubiquitination and acetylation, serve crucial roles in regulating SLUG function and stability (2,30,31). Ubiquitination serves a primary role in governing the post-translational stability of SLUG, directly impacting protein levels within cells (32,33). Several

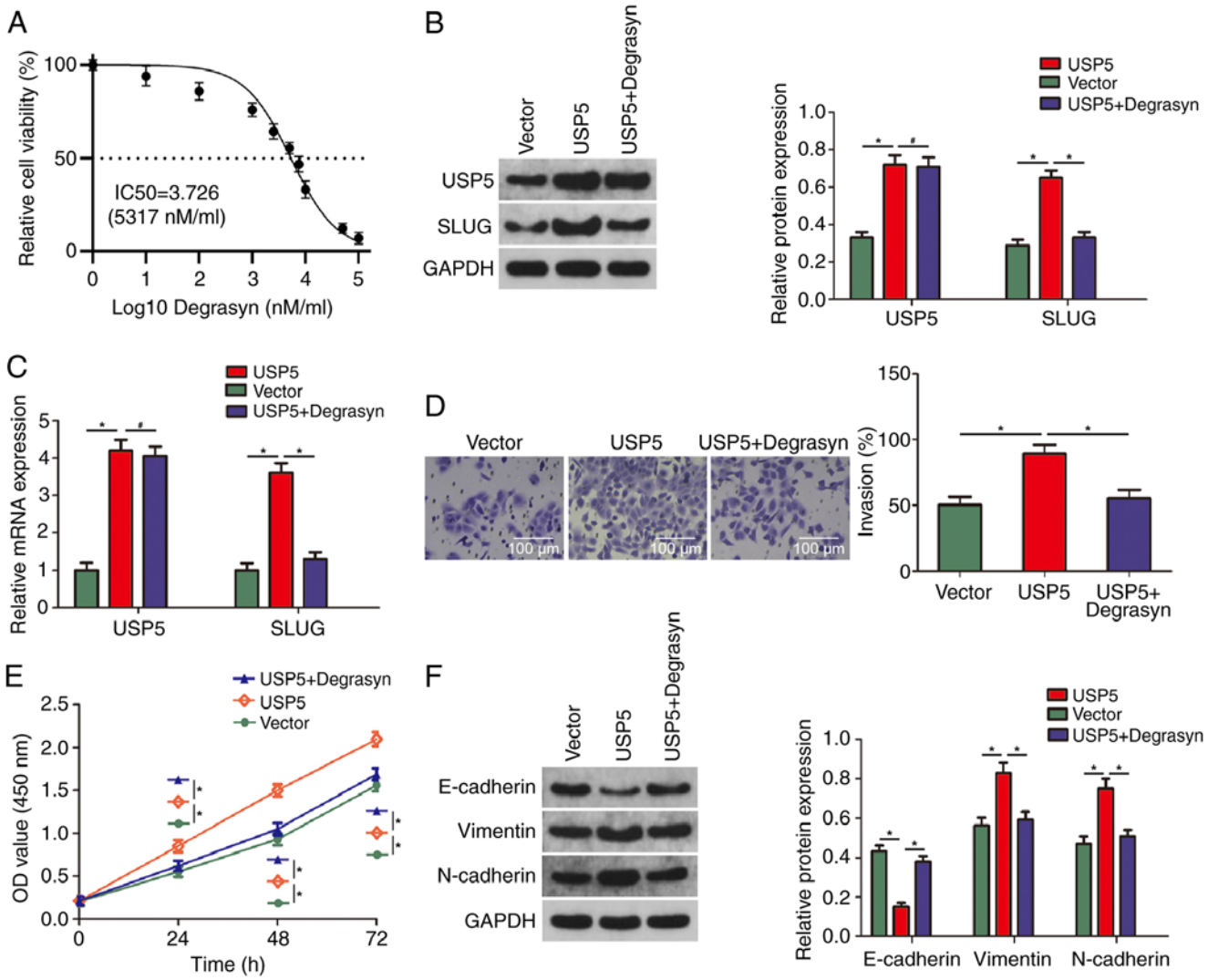

Figure 6. Pharmacological inhibition of USP5 suppresses bladder cancer progression. (A) Cell viability of T24 cells at different Degrasyn concentrations. (B) Protein expression and (C) mRNA levels of SLUG in the USP5 overexpression T24 cell line with or without Degrasyn treatment (USP5 inhibitor, 2 μg/ml). Results of the (D) Transwell and (E) cell proliferation assays in the USP5 overexpression T24 cell line with or without Degrasyn treatment (2 μg/ml). (F) Protein expression levels of epithelial-mesenchymal transition markers (E-cadherin, N-cadherin and vimentin) in the USP5 overexpression T24 cell line with or without Degrasyn treatment (2 μg/ml). *P<0.05; #P>0.05. USP5, ubiquitin-specific proteinase 5; SLUG, Snail family zinc finger 2; IC_{50}, half maximal inhibitory concentration; OD, optical density.

studies have identified multiple E3 ubiquitin ligases responsible for ubiquitinating SLUG (34,35). Notably, research by Li *et al* (32) reported the role of USP20 in modulating SLUG protein stability in breast cancer. Additionally, USP10 has been implicated in regulating SLUG stability, thereby influencing tumor EMT (36). The findings of the present study indicate that USP5 modulates SLUG protein stability, thereby contributing to the proliferation, invasion and EMT processes in bladder cancer.

USPs are implicated in a myriad of pathological processes within malignant tumors, rendering them promising targets for drug development (37). Several inhibitors targeting USP5 have been developed for treating several human cancers, including PYR-41 (38), WP1130 (39) and Degrasyn (40). The present study underscores USP5 as a potential therapeutic target for bladder cancer treatment. Furthermore, the present study assessed the therapeutic efficacy of USP5 inhibitors in bladder cancer. The experimental findings revealed that Degrasyn, an inhibitor of USP5, significantly impeded the proliferation, invasion and EMT progression of bladder cancer cells *in vitro*.

However, the present study has certain limitations. As a research subject, the human bladder cancer T24 cell line does not fully represent the real biological characteristics of bladder cancer. Furthermore, *in vitro* cellular experiments do not fully represent the role of USP5 in bladder cancer *in vivo*. Further *in vitro* studies of other cell lines and *in vivo* studies in mice are essential to evaluate the effectiveness of USP5-related mechanisms in the treatment of bladder cancer.

In conclusion, the experimental findings of the present study indicate a significant association between USP5 expression and poor prognosis, as well as a higher clinicopathological stage, in bladder cancer. By elucidating its oncogenic mechanisms, the present study identified that USP5 may promote bladder cancer progression through the deubiquitination of SLUG,

a crucial transcription factor implicated in EMT. Furthermore, the findings underscore the potential of USP5 as a novel therapeutic target in the management of bladder cancer.

Acknowledgements

Not applicable.

Funding

The present work was funded by the Natural Science Foundation for Colleges and Universities of Anhui Province Department of Education (grant no. KJ2019A0308).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

BH designed the research. QW and TL participated in the research design and implementation. BL participated in the analysis and interpretation of data. BH and BL confirm the authenticity of all the raw data All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committees of the First Affiliated Hospital of Bengbu Medical University (Bengbu, China; approval no. 2020083). All patients submitted written informed consent for tissue use.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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