

Deubiquitinating enzyme USP20 is a positive regulator of Claspin and suppresses the malignant characteristics of gastric cancer cells

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Abstract. The aim of the present study was to investigate the clinical significance, the biological function and the mechanisms of USP20 in gastric cancer. The expression of USP20 in 89 pairs of primary gastric cancer and peritumoral gastric tissues specimens were measured by immunohistochemistry. The correlation of USP20 expression with the survival and the clinicopathological characteristics of patients were analyzed. Moreover, the underlying mechanisms of ectopic USP20 expression and its impact on GC cells were also investigated. We found that the expression of USP20 is relatively low in GC tissues and negatively correlated with tumor size, tumor invasion and TNM staging. High expression of USP20 in GC predicted longer survival. Experimentally, small interfering RNA-mediated knockdown of USP20 expression significantly promoted cell proliferation, accelerated G1-S phase transition and attenuated the autophagy activity. Overexpression of USP20 led to the inhibition of proliferation, G1-S cell cycle transition delay and autophagy activation. Mechanistically, we confirmed that silencing the expression of USP20 in GC cells could reduce Claspin protein levels without altering Claspin mRNA levels, which is involved in the antitumor activity of USP20. Furthermore, the expression level of Claspin was relatively higher in peritumoral tissue than that of GC tissues and higher expression of Claspin in GC was also correlated with good prognosis of patients. Given its pivotal role in gastric tumorigenesis and progression, USP20 functioned as the tumor suppressor in GC and possessed promising value to be a therapeutic target for GC.

Introduction

Gastric cancer (GC) is the fifth most common malignancy and the third leading cause of cancer death in the world, with 951,600 new cases and 723,100 deaths per year (1). Almost half of the new cases occur in Eastern Asia, and mainly in China (423,500 cases) (2). Radical gastrectomy offers the best opportunity for curative therapy, while most patients are diagnosed as advanced and unresectable GC thus losing the opportunity for surgery. For these patients, the efficacy of chemotherapy or radiotherapy even with targeted therapy are still limited (3). To break through the bottleneck of treatment, investigating the molecular mechanism of GC initiation and progression are urgently needed.

Post-translational modification by ubiquitination and deubiquitination could modulate functions of target proteins, fate of proteins and intracellular mechanisms (4). Therefore, the modification of enzyme-mediate processes via E3 ligases and deubiquitinating enzymes (DUBs) is crucial for cellular homeostasis and cell viability (5), and the DUBs can be further categorized into five subtypes: the ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), Machado-Joseph disease protein domain proteases (MJDs), ovarian tumor proteases (OTUs) and JAMM motif proteases. Among DUBs, USPs is the largest group which contain more than 50 members (6). Currently, many studies have found that various DUBs serve as a tumor suppressor or an oncogene depending on their regulation of multiple biological processes such as DNA damage response, cell cycle regulation, chromatin remodeling and modulating kinase cascades of signal transduction pathways that are frequently altered in cancer (7), such as the ectopic expressions of USP2, USP7 or USP33 have an impact on tumor progression (8-10). According to the function and importance of DUBs, USP20, the homologue of USP33, also plays a role in the cellular system through its deubiquitinating capacity, and it was reported to regulate the stability and function of oncoproteins such as PKM2, HIF-1 α , Rad17 and TRAF6 (11-14). Moreover, the silencing of USP20 expression promoted xenograft tumor growth in colon cancer HCT116 cells (15). However, there

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are still no published reports that have described the role of USP20 in GC.

In the present study, we investigated the clinical correlation of USP20 in GC specimens and its biological functions on GC, and our results provided evidence for the tumor suppressor role of USP20 in GC and the possibility of USP20 being the target against GC.

Materials and methods

Cell culture and transfection. Human gastric cancer cell lines AGS, BGC-823, MKN74, SNU-16, MGC-803, MKN45, NCI-N87, SGC-7901, SNU-1 and KATO III, and normal GES-1 gastric mucosal cells were obtained from the Shanghai Institute of Digestive Surgery (Shanghai, China). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C with 5% CO₂ and saturated humidity.

The full-length complementary cDNA of human USP20 was synthesized by Vigene Biosciences (Jinan, China) and cloned into the expression vector pENTER. Plasmid vectors for transfection were prepared using EndoFree Plasmid Miniprep kits (Biomiga). Three small interfering RNAs (siRNA) toward USP20 were synthesized by Shanghai GenePharma, Co., Ltd., (Shanghai, China) and merged into one siRNA pool. The siRNA target sequences were as follows: siRNA-1680: CAGCCAUCUACCAGAAUGUTT; siRNA-1985: GAGGUGAUGUACUCAUUCATT; and siRNA-2439: CCCACAAUACCACUACAUTT. These sequences of siRNA were analyzed using BLAST to avoid non-specific silencing of unrelated genes. Three groups were designed in the present study: blank control, negative siRNA control/empty vector control and USP20-specific siRNA/pEnter-USP20. Blank control groups were cultured with only DMEM medium without any transfection reagents or siRNA. Negative control groups were treated with negative siRNA or empty vector that did not match any known human coding cDNA. Lipofectamine 2000 was used for the transfection, and together with siRNAs or plasmid were transfected into cells according to the manufacturer's instructions. All the *in vitro* experiments were performed in triplicate.

Cell proliferation. Cell Counting kit-8 (CCK-8) (Dojindo Laboratories, Inc., Kumamoto, Japan) was used to detect cell proliferation. A total of 200 μ l volume of 500 cells/well was seeded in 96-well culture plates. Cells were placed in 5-wells and then were cultured at 37°C for 0, 24, 48, 72, 96 and 120 h, respectively. Then, 20 μ l of CCK-8 reagent was added to each well and the plates were cultivated at 37°C for 2 h in a humidified environment with 5% CO₂. The absorbance at 450 nm was read using microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA) to calculate the number of vital cells in each well. Cell growth curves were drawn.

Tissue microarrays (TMAs) and immunohistochemistry staining (IHC). TMAs were purchased from Shanghai Biochip Co., Ltd., (Shanghai, China). The histopathological diagnosis was determined by the World Health Organization criteria. Tumor staging was defined according to the 6th edition

of the tumor-node-metastasis (TNM) classification of the International Union Against Cancer. Overall survival (OS) was termed as the interval between the dates of surgery and death or final follow-up.

Immunohistochemistry staining of paraffin sections were manufactured on 4 μ m-thick slides, followed by EnVision two-step procedure of Dako REAL™ EnVision™ detection system (Dako, Carpinteria, CA, USA). After antigen retrieval, the slice was incubated with the primary antibodies USP20 (1:250; Proteintech Group, Chicago, IL, USA) and Claspin (1:1500; Proteintech Group) overnight at 4°C, followed by incubation with the HRP labeled secondary antibodies at 37°C for 0.5 h and slide was visualized by diaminobenzidine.

Evaluation of immunohistochemical variables. USP20 proteins were localized in the cytoplasm and nucleus of cell and stained as brownish granules, and Claspin protein were localized in the nucleus of the cell and stained as brownish granules. The expression status of USP20 and Claspin was scored using a 4-point scale (0-4) based on the amount of positive cells and intensity of the staining under 5 random high-power fields. Score for percentage: <5% (0), 5-25% (1), 25-50% (2), 50-75% (3) and >75% (4); for intensity: no staining (0), light brown (1), brown (2) and dark brown (3). The final score of each specimen was calculated by multiplying staining intensity score with score of stained cells, score of ≤ 2 , 3-4, 6-8 and 9-12 were defined as negative (-), weak (+), moderate (++) and strong (+++), respectively. We defined 5 as the cut-off value to divide the high and low expression of USP20 and Claspin, GC patients with low or high expression were classified as USP20^{low} or USP20^{high} and Claspin^{low} or Claspin^{high}, respectively.

Quantitative reverse transcription-polymerase chain reaction (RT-qPCR). Total RNA was isolated from different cell lines using TRIzol reagent method. Equal amounts of RNA were reverse transcribed into cDNA following the protocol of Applied Biosystems. Primers for RT-qPCR were USP20: F-TGGGCTAGTCTGTAAGTCGC, R-GGGACCTGCTCTT TGGATGTT; Claspin: F-AGAATGCCAGTCGCCCTATG, R-GCTTCGCGTCAATCCTGAAG; GAPDH: F-AATGGG CAGCCGTTAGGAAA, R-GCCCAATACGACCAATCA GAG; GAPDH was used as an internal control. Quantitative mRNA expression was performed by ABI Prism 7900HT sequence detection system (Applied Biosystems), and relative mRNA expression was measured based on the Ct values, which were revised for GAPDH expression according to the comparative Ct method (16). All experiments were carried out in triplicate.

Western blot analysis. MKN45, NCI-N87, BGC-823 and MGC-803 cells (5×10^5 /well) were cultured overnight in 6-well plates and transfected as described above. Two days later, the cells were harvested using mammalian protein extraction reagent (Pierce, Rockford, IL, USA) mixed with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The cell lysates (50 μ g/lane) were separated on 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After blocking with 5% non-fat milk in PBST buffer for 2 h, the membranes were incubated overnight at 4°C with the primary

antibodies for USP20 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Claspin (1:1,000; Proteintech Group), ATG5 (1:1,000; Cell Signaling Technology, Boston, MA, USA), LC3 (1:1,000; Cell Signaling Technology) or GAPDH (1:2,500; Santa Cruz Biotechnology) and GAPDH was used as loading control. Secondary antibodies (1:10,000; LI-COR Biosciences, Lincoln, NE, USA) and infrared imaging system (LI-COR Biosciences) were applied to visualize the protein bands.

Flow cytometric analysis. For cell cycle analysis, the MKN45, NCI-N87, BGC-823 and MGC-803 cells were seeded in 6-well plates at 5×10^5 /well; 48 h after the transfection, the cells were fixed in 70% ethanol at 4°C for 24 h and stained with 300 μ l propidium iodide (PI) (BD Biosciences, Bedford, MA, USA). Then, the cell cycle distribution was analyzed by FACS (Becton-Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis. Data were presented as mean \pm standard deviation (SD). Quantitative variables were analyzed using independent-samples t-test between two groups or one-way analysis (ANOVA) among multiple groups. The categorical variable comparisons were tested by the Chi-square test or the Fisher's exact test. The Kaplan-Meier method was used to compare the survival of patients in the groups of high and low USP20 expression or high and low Claspin expression, and the differences between the survival curves were tested using the log-rank test. The Cox proportional hazards regression model was used to perform the univariate and multivariate survival analysis. For the multivariate model, 0.20 was used as the cut-off P-value to select the analyzed factors from the univariate analysis data. We used a forward stepwise Cox regression model to find independent prognostic factors. All the statistical analyses were executed by the SPSS 19.0 for Macintosh (SPSS, Inc., Chicago, IL, USA). Two-tailed $P < 0.05$ were considered statistically significant.

Results

USP20 protein is expressed at low level in human gastric cancer tissues. We investigated the USP20 protein expression in gastric cancer tissues by immunohistochemistry staining (IHC). Gastric cancer and paired peritumoral gastric tissues from 89 patients were stained. Representative photomicrographs of USP20 staining are shown in Fig. 1A. USP20 were mainly located in the cytoplasm and nucleus of cells. The IHC scores of USP20 were relatively higher in peritumoral gastric tissues than that of gastric cancer (Fig. 1B). Moreover, a significant correlation was found between the low expression of USP20 with tumor size (≥ 57 cm³, $P < 0.0001$), tumor invasion (T3+T4, $P < 0.0001$) and late TNM staging (III+IV, $P = 0.036$). Other parameters including age, gender, lymph node metastasis, lymphatic invasion and tumor location were not associated with USP20 expression level (Table I). These data demonstrated that USP20 expression was relatively lower in this training cohort of GC patients which show more malignant characteristics according to clinicopathological features.

USP20 expression levels correlate with the prognosis in GC patients. To investigate the prognostic significance

Table I. Summary of the data on the expression of USP20 in clinicopathological features of 89 cases of gastric cancer.

Category	No. of patients (%)	USP20		P-value
		Low	High	
Age (years)				
<65	39 (43.8)	18 (48.6)	21 (40.4)	0.439
≥ 65	50 (56.2)	19 (51.4)	31 (59.6)	
Gender				
Male	61 (68.5)	24 (64.9)	37 (71.2)	0.529
Female	28 (31.5)	13 (35.1)	15 (28.8)	
Tumor size (cm³)				
<57	50 (56.2)	11 (29.7)	39 (75.0)	<0.0001
≥ 57	39 (43.8)	26 (70.3)	13 (25.0)	
Lymph node metastasis				
Negative	25 (28.1)	9 (24.3)	16 (30.8)	0.505
Positive	64 (71.9)	28 (75.7)	36 (69.2)	
Lymphatic invasion				
Negative	72 (80.9)	32 (86.5)	40 (76.9)	0.258
Positive	17 (19.1)	5 (13.5)	12 (23.1)	
Tumor invasion				
T1+T2	45 (50.6)	2 (5.4)	43 (82.7)	<0.0001
T3+T4	44 (49.4)	35 (94.6)	9 (17.3)	
TNM staging				
I+II	43 (48.3)	13 (35.1)	30 (57.7)	0.036
III+IV	46 (51.7)	24 (64.9)	22 (42.3)	
Tumor location				
Gastric fundus	18 (20.2)	7 (18.9)	11 (21.2)	0.546
Gastric corpus	28 (31.5)	14 (37.8)	14 (26.9)	
Pylorus	43 (48.3)	16 (43.2)	27 (51.9)	

of USP20 in GC, we performed the correlation of USP20 with the patient survival using Kaplan-Meier analysis. The results revealed that the low expression of USP20 was closely correlated with worse overall survival (medium survival, 25 months vs. 45 months, $P = 0.005$; Fig. 1C). In the training cohort, the 3- and 5-year survival rates of the USP20^{high} patients were significantly higher than those of the USP20^{low} group (48.1 vs. 21.6%, and 44.2 vs. 18.9%, respectively; Fig. 1C). The relationship between USP20 expression level and the outcome of the patients was further investigated using univariate and multivariate Cox regression models. In the univariate analysis, age, tumor size, tumor invasion and TNM staging were associated with OS. In multivariate analysis, age, tumor size and TNM staging were associated with OS. Univariate and multivariate analyses revealed that USP20 expression serves as an independent risk factor for OS ($P = 0.003$, HR=0.459; $P = 0.008$, HR=0.525, respectively; Table II). These findings indicated that the loss of USP20 expression have a deep impact on GC development and progression and is a potential biomarker for GC.

Table II. Univariate and multivariate analyses of factors associated with survival in 89 cases of gastric cancer.

Variable	Univariate overall survival			Multivariate overall survival		
	Hazard ratio	95% Confidence limits	P-value	Hazard ratio	95% Confidence limits	P-value
Age (years)						
<65 (n=39)	1			1		
≥65 (n=50)	1.683	1.038-2.732	0.035	1.670	1.040-2.682	0.034
Gender						
Male (n=61)	1					
Female (n=28)	1.036	0.624-1.719	0.893			
Tumor size (cm ³)						
<57 (n=50)	1			1		
≥57 (n=39)	2.324	1.256-4.301	0.007	2.004	1.199-3.35	0.008
Lymph node metastasis						
Negative (n=25)	1					
Positive (n=64)	1.975	0.847-4.604	0.115			
Lymphatic invasion						
Negative (n=72)	1					
Positive (n=17)	0.776	0.428-1.406	0.403			
Tumor invasion						
T1+T2 (n=45)	1					
T3+T4 (n=44)	2.427	0.978-6.024	0.056			
TNM staging						
I+II (n=43)	1			1		
III+IV (n=46)	3.115	1.307-7.425	0.01	3.145	1.92-5.15	<0.0001
Tumor location						
Gastric fundus (n=18)	1					
Gastric corpus (n=28)	0.898	0.459-1.755	0.752			
Pylorus (n=43)	0.922	0.539-1.577	0.767			
USP2						
Low (n=37)	1			1		
High (n=52)	0.459	0.274-0.77	0.003	0.525	0.327-0.843	0.008

Claspin expression level is correlated with USP20 in GC tissues and predicts the prognosis of GC patients. Previous studies showed that Claspin is a major target for USP20-mediated tumor suppression, and overexpression of which could attenuate tumorigenesis induced by USP20 depletion (17). To further explore the relationship between USP20 and Claspin, we confirmed the claspin expression in the same GC tissues from 89 patients and matched non-tumor tissues. Not unexpectedly, the IHC scores of Claspin were also higher in peritumoral gastric tissues than that of GC tissues ($P < 0.0001$; Fig. 2A), and a scatter plot of USP20 and Claspin expression in GC tissues revealed a positive correlation between USP20 and Claspin at the protein level ($R = 0.266$, $P = 0.0113$; Fig. 2B). The representative microphotographs of USP20 and Claspin staining in GC tissues and adjacent non-tumor tissues are presented in Fig. 2C, and it was demonstrated that Claspin is located in the nucleus of the cells.

To confirm whether Claspin has influence on the prognosis of patients, we performed correlation of Claspin with

the patient survival using Kaplan-Meier analysis. The results revealed that low expression of Claspin was closely correlated with worse overall survival (medium survival, 17 months vs. 22 months, $P = 0.033$; Fig. 3A). Moreover, GC patients co-expressing high levels of USP20/Claspin had the best prognosis in terms of overall survival ($P = 0.0183$; Fig. 3B). These results demonstrated that expression of USP20 and/or Claspin is an independent parameter for predicting the prognosis of GC patients.

Expression of USP20 in gastric cancer cell lines. We next examined the USP20 expression in a series of GC cell lines (MGC-803, NCI-N87, MKN45, BGC-823, KATO III, SGC-7901, AGS, SNU-1, SNU-16 and MKN74) and normal gastric mucosal cells GES-1. Firstly, RT-qPCR was performed to analyze the USP20 mRNA expression in GC cell lines and GES-1 (Fig. 3C) and the protein levels of USP20 in the above cells were investigated (Fig. 3D). We found that USP20 expression was relatively lower in all GC cell lines than GES-1. We

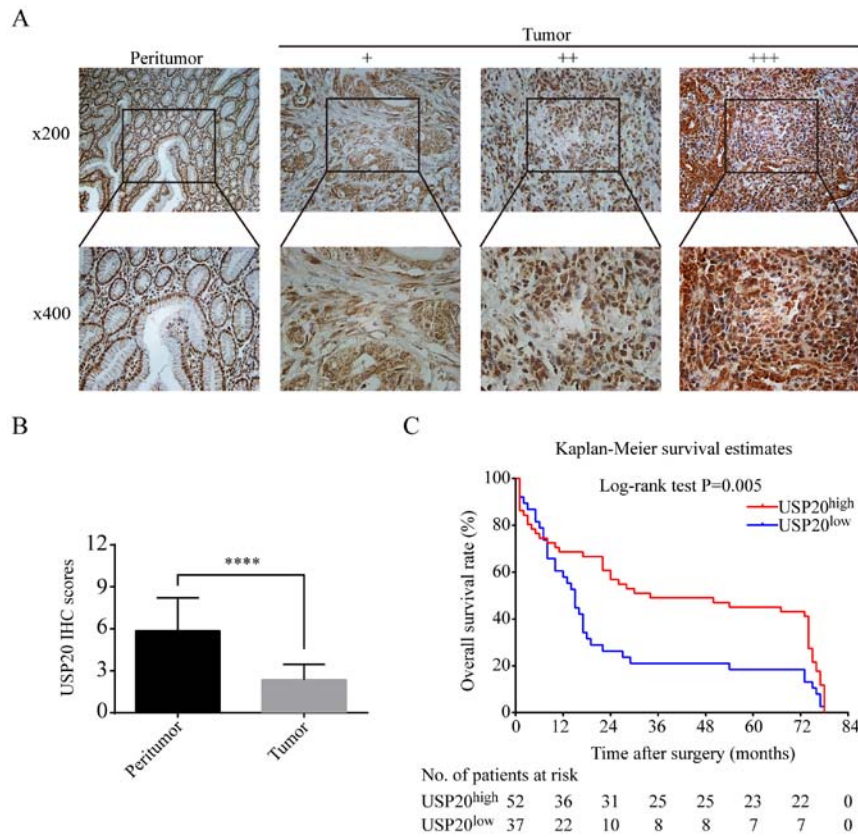


Figure 1. Expression of USP20 protein levels in human gastric cancer and paired peritumoral gastric tissues. (A) Representative images of USP20 staining in peritumor tissues and tumor tissues of the different staining patterns are presented here and are graded from + to +++ (magnification, x200 and x400). (B) Analysis of the IHC scores of USP20 expression levels in gastric cancer specimens compared with peritumoral gastric tissues specimens. ****P<0.0001. Error bar indicates standard deviation. (C) Kaplan-Meier analysis and the log-rank test identified USP20 significantly associated with gastric cancer patient prognosis.

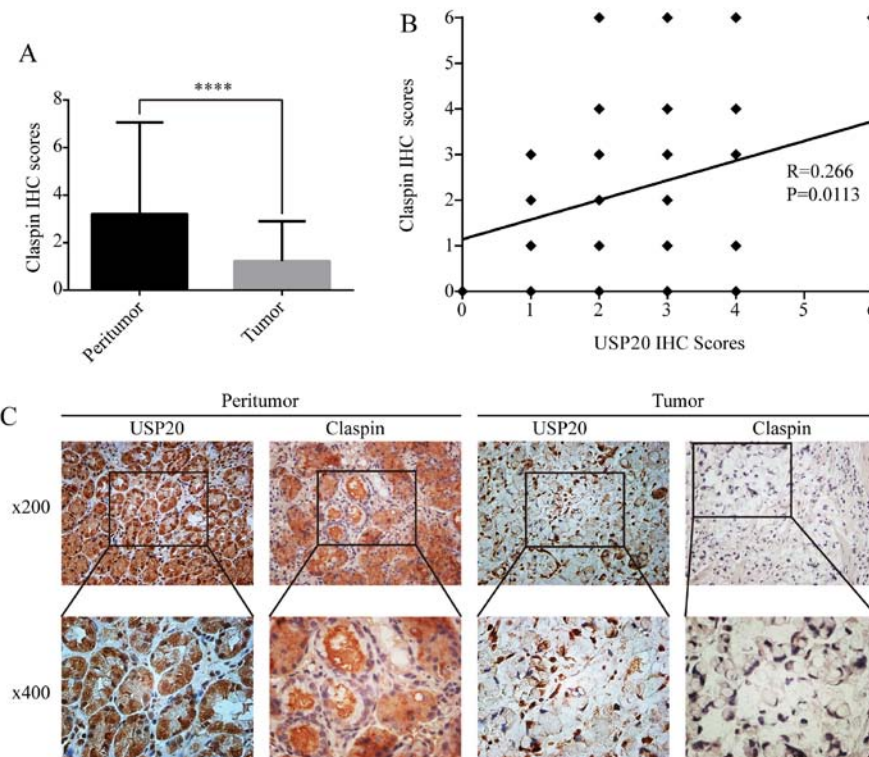


Figure 2. The correlation of USP20 with the expression of Claspin in the tissues of gastric cancer patients. (A) Analysis of the IHC scores of Claspin expression levels in gastric cancer specimens compared with adjacent non-tumor tissues specimens. ****P<0.0001. Error bar indicates standard deviation. (B) Scatter plot of USP20 and Claspin expression in GC tissues. (C) The expression pattern of USP20 and Claspin in primary tumor tissues and matched non-tumor tissues from the same patient was examined by immunohistochemistry. (Original magnification, x200 and x400).

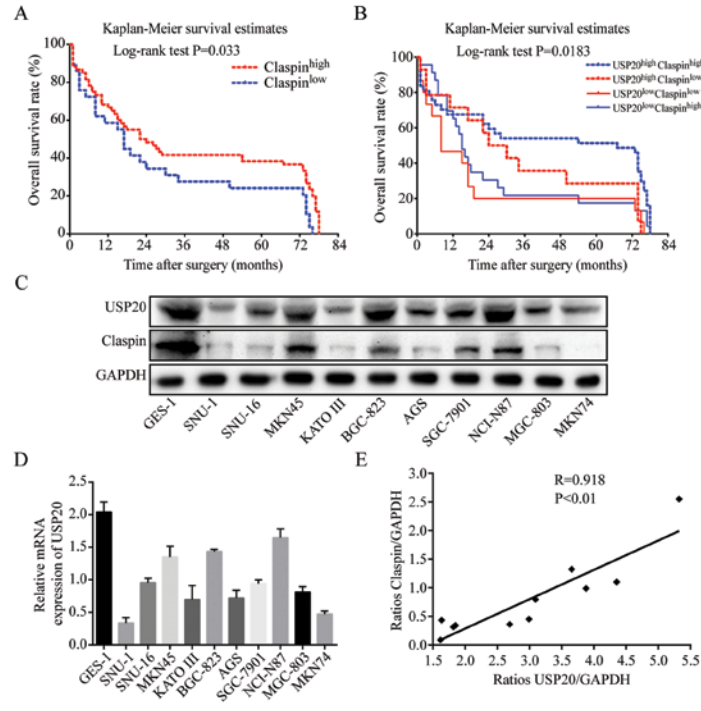


Figure 3. Expression of USP20 and Claspin in GC cell lines and prognostic significance in gastric cancer patients (training cohort, n=89). (A and B) Kaplan-Meier analysis and the log-rank test identified USP20 and/or Claspin as significant associated with the gastric cancer patient prognosis. USP20 protein (C) and mRNA (D) levels in ten GC cell lines and normal gastric epithelial cell line GES-1. GAPDH was used as a loading control. Error bar indicates standard deviation from three independent experiments. (E) Scatter plot of USP20 and Claspin protein expression in GC cell lines.

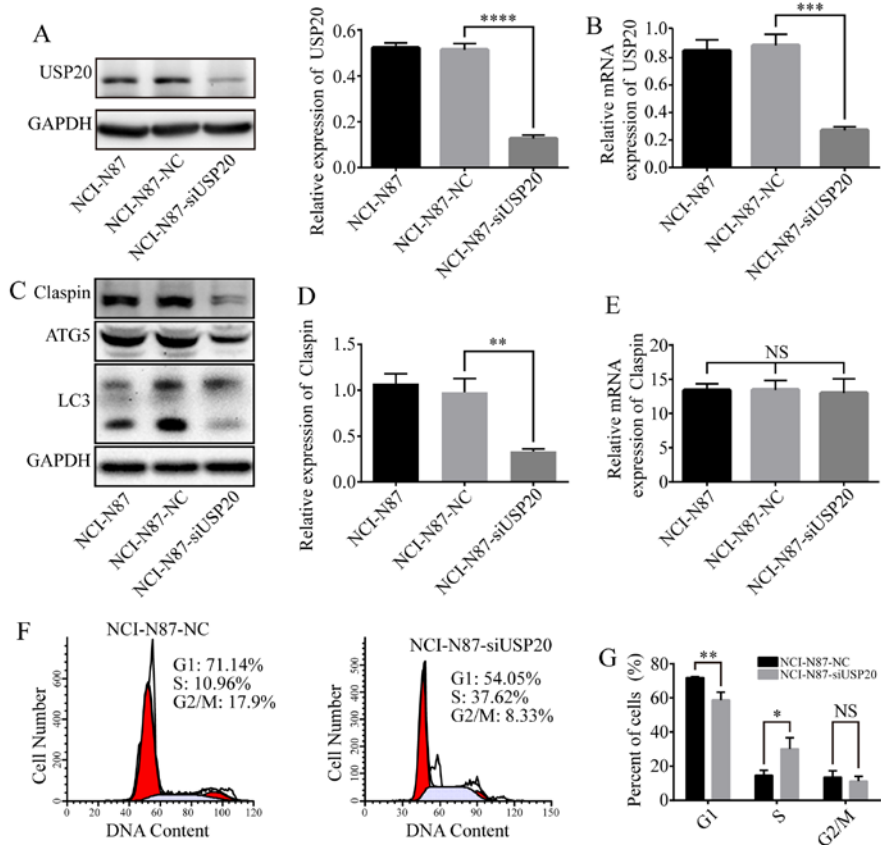


Figure 4. Effect of altered USP20 expression on NCI-N87 cell lines. (A and B) USP20 expression in NCI-N87 cells was modified by siRNA interference and was verified by immunoblotting and RT-qPCR. (C-E) Silencing the expression of USP20 in NCI-N87 cells resulted in the reduction of Claspin protein levels without altering Claspin mRNA levels, quantitative western blot analysis results obtained using densitometric analysis and the mRNA expression levels which were standardized according to GAPDH. The expression of ATG5 and LC3 I/II conversion, which are autophagy markers, were also downregulated and reversed vs. normal cells and control cells treated with scrambled siRNA. (F and G) NCI-N87 cells treated with USP20 siRNA accelerated the G1-S phase transition. Error bar indicates standard deviation from three independent experiments. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

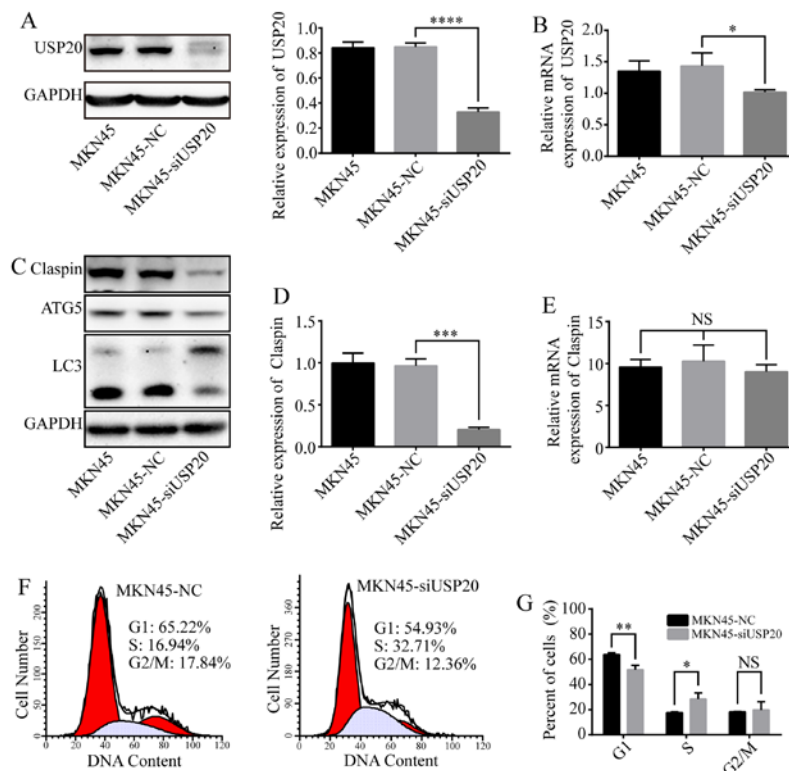


Figure 5. Effect of altered USP20 expression on MKN45 cell lines. (A and B) USP20 expression in MKN45 cells was modified by siRNA interference and was verified by immunoblotting and RT-qPCR. (C-E) Silencing the expression of USP20 in MKN45 cells resulted in the reduction of Claspin protein levels without altering Claspin mRNA levels, quantitative western blot analysis results obtained using densitometric analysis and the mRNA expression levels were standardized according to GAPDH. The expression of ATG5 and LC3 I/II conversion, which are the autophagy markers, were also downregulated and reversed vs. normal cells and control cells treated with scrambled siRNA. (F and G) MKN45 cells treated with USP20 siRNA could accelerate the G1-S phase transition. Error bar indicates standard deviation from three independent experiments. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$.

confirmed that Claspin, the stability of which was modulated by the deubiquitination role of USP20 (15,17), was positively correlated with USP20 expression at a statistically significant level ($R=0.918$, $P<0.01$; Fig. 3C and E). These data revealed that USP20 expression is relatively low in GC cell lines which is consistent with that of GC tissues, moreover, it was supposed that USP20 might play a role in GC cells via mediating the stability of Claspin.

Effects of altered USP20 expression on Claspin expression and cell cycle in GC cells. Since the USP20 expression of MKN45, NCI-N87 and BGC-823 were relatively higher in all GC cell lines (Fig. 3C and D), we treated these three cell lines with siUSP20 to clarify the effect of silenced USP20 expression on Claspin expression and cell cycle of GC cells. siUSP20 led to a significant reduction of USP20 protein and mRNA levels in NCI-N87, MKN45 and BGC-823 (Figs. 4A and B, 5A and B and 6A and B). We then found that the Claspin expression was changed at protein level without altering Claspin mRNA levels (Figs. 4C-E, 5C-E and 6C-E). These results revealed that USP20-mediated Claspin downregulation did not occur at the gene transcription level, just as the prior study reported that USP20 deubiquitinate and stabilize Claspin (15).

As a previous study has shown, USP20 could regulate cell-cycle checkpoint activation via targeting Claspin (17). Thus, we performed the cell cycle assays to investigate the role of USP20 in the cell cycle. As shown in Fig. 4F, the silencing of USP20 expression accelerated the G1-S cell cycle transition which is

accompanied by decrease in the percentage of G1 cells (down to 54.05%) and increase in the percentage of cells in the S phase (up to 37.62%), and the tendency was also consistent with MKN45-siUSP20 cells and BGC-823-siUSP20 cells ($P<0.05$; Figs. 5F and 6F).

To further illustrate the role of USP20 in GC cells, we successfully overexpressed USP20 gene in MGC-803 cells with relatively low USP20 expression background (Fig. 7A and B), although the mRNA and protein level of USP20 in the SNU-1 and KATO III were much lower than that of MGC-803, neither was anchorage-dependent or difficult to transfect. As shown in Fig. 7C-E, overexpression of USP20 increased the protein level of Claspin without the change of mRNA level. In terms of cell cycle, we found that the MGC-803-USP20 cells have 70.14% of cells in the G1 phase and 20.01% in the S phase, while G1 phase of the MGC-803-NC cells occupied 45.52% and S phase occupied 39.03% respectively (Fig. 7F and G), which demonstrated that overexpression of USP20 could delay G1-S cell cycle transition.

Effect of silenced USP20 on autophagy in GC cells. A prior study revealed that silencing of USP36, one of the deubiquitinating enzymes, could trigger selective autophagy via a p62-dependent pathway (18). Whether silencing of USP20 could affect the autophagy in GC cells was assessed by western blotting to investigate the expression of ATG5 and LC3 I/II conversion in mock, negative siRNA control and siUSP20 cells. We found that ATG5 expression and LC3 I/II conversion

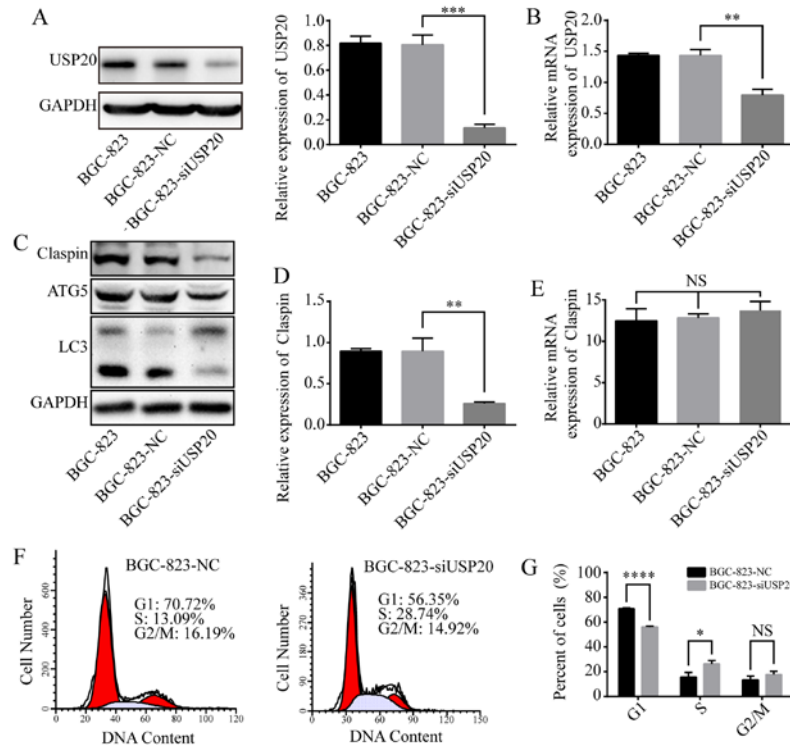


Figure 6. Effect of altered USP20 expression on BGC-823 cell lines. (A and B) USP20 expression in BGC-823 cells was modified by siRNA interference and was verified by immunoblotting and RT-qPCR. (C-E) Silencing the expression of USP20 in BGC-823 cells resulted in the reduction of Claspin protein levels without altering Claspin mRNA levels, quantitative western blot analysis results obtained using densitometric analysis and the mRNA expression levels which were standardized according to GAPDH. The expression of ATG5 and LC3 I/II conversion, which are the autophagy markers, were also downregulated and reversed vs. normal cells and control cells treated with scrambled siRNA. (F and G) BGC-823 cells treated with USP20 siRNA could accelerate the G1-S phase transition. Error bar indicated standard deviation from three independent experiments. *P<0.05; **P<0.001; ****P<0.0001.

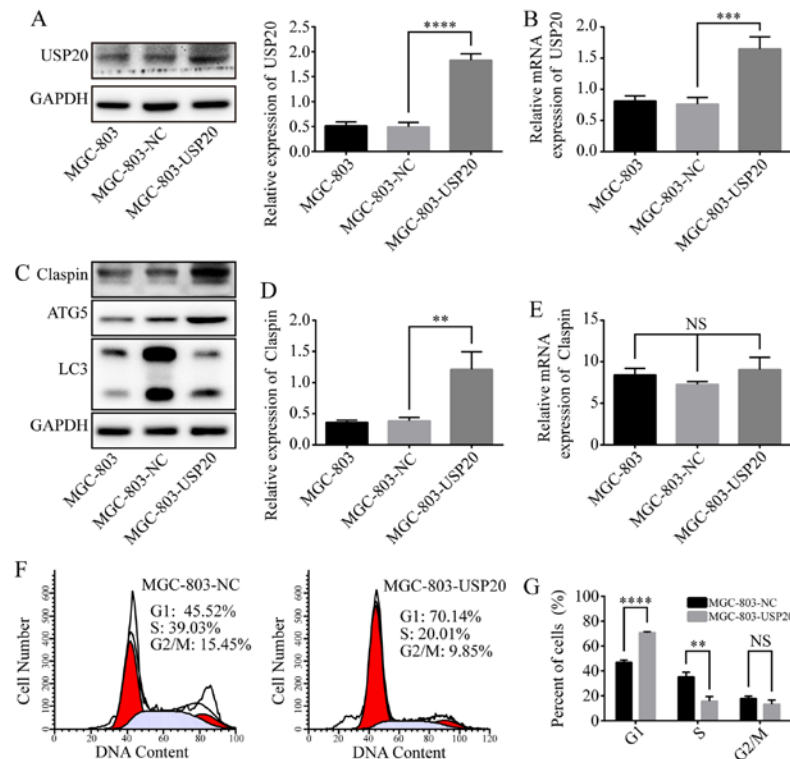


Figure 7. Effect of altered USP20 expression on MGC-803 cell lines. (A and B) USP20 expression in MGC-803 cells was modified by pEnter-USP20 plasmid and was verified by immunoblotting and RT-qPCR. (C-E) The overexpression of USP20 in MGC-803 cells resulted in the upregulation of Claspin protein levels without altering Claspin mRNA levels. Quantitative western blot analysis results obtained using densitometric analysis and the mRNA expression levels which were standardized according to GAPDH. The expression of ATG5 and LC3 I/II conversion, which are the autophagy markers, were also upregulated and enhanced vs. normal cells and control cells treated with empty vector. (F and G) MGC-803 cells treated with pEnter-USP20 could delay the G1-S phase transition, Error bar indicates standard deviation from three independent experiments. **P<0.01; ***P<0.001; ****P<0.0001.

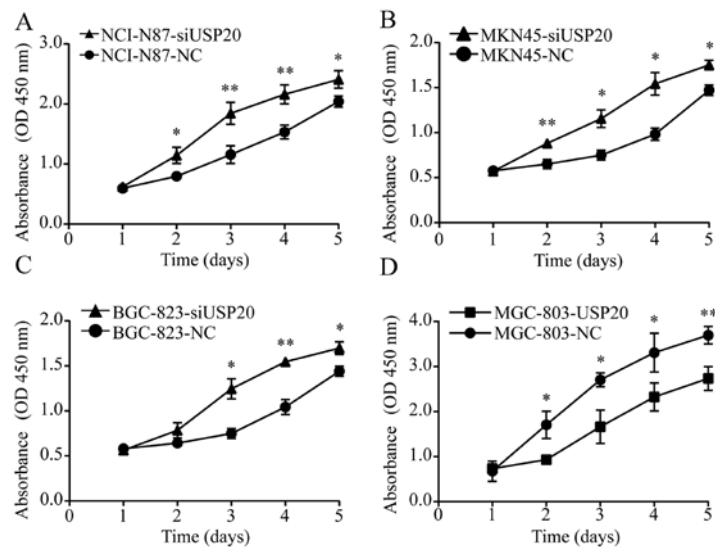


Figure 8. The altered expression of USP20 modulates cell proliferation. (A-C) NCI-N87, MKN45 and BGC-823 cells were transfected with siCTR or siUSP20. (D) MGC-803 cells were transfected with empty vector or pEnter-USP20, and the abilities of cell proliferation was detected by CCK-8 assay. Error bar indicates standard deviation from three independent experiments. * $P < 0.05$. ** $P < 0.01$.

were inhibited in siUSP20 cells when compared with the other two groups (Figs. 4C, 5C and 6C). On the contrary, autophagy activity was enhanced by the USP20 overexpression (Fig. 7C). These results demonstrated that silencing of USP20 could prevent autophagy activation, which is contrary to the effect of USP36 on autophagy.

Silencing of USP20 increases proliferation in GC cells. Previous studies reported that depletion of USP20 by lentiviral shRNA could enhance tumorigenesis in xenograft mouse models of colon cancer cells (15). To investigate the role of USP20 in GC cells, we performed a cell proliferation assay in monolayer culture. As shown in Fig. 8A-C, silencing of USP20 significantly promoted cell growth in monolayer culture, and further experimental results revealed that the proliferation ability of MGC-803-USP20 cells were lower than that of MGC-803-NC cells (Fig. 8D). These finding revealed that USP20 could modulate GC cell proliferation.

Discussion

USP20 is not merely a deubiquitinating enzyme, but also a substrate of the von Hippel-Lindau tumor suppressor protein (pVHL). A prior study revealed that the mutation of the pVHL gene is strongly associated with many kinds of tumors such as haemangioblastoma, pheochromocytoma and clear-cell renal cell carcinoma (ccRCC) (19). Currently, a study found that the high frequency of the 3-bp deletion in the USP20 gene in Korean population, which mean a biased susceptibility of Korean individuals to certain cancer types, and the potential mechanisms of carcinogenesis caused by mutant USP20 are still unknown (20). Furthermore, searching public databases of the Sanger Cosmic and the Cancer Genome Atlas, frequent point mutations and deletions of USP20 are found to exist in a variety of malignancies (13). Moreover, USP20 has been confirmed involved in various cellular mechanisms by

impeding Tax- and interleukin 1 β (IL-1 β)-induced NF- κ B activation by targeting Tax and TRAF6 for deubiquitination, which results in slower cell growth in ATL2 cells (14), enhancing HIF-1 α -induced hypoxia response element (HRE) luciferase activity and VEGF protein expression via salvaging ubiquitinated HIF-1 α from proteasomal degradation (12), and regulating β 2 adrenergic receptor (β 2AR) recycling and resensitization (21). As Rad17 was implicated in carcinogenesis as a tumor suppresser gene that play a role in a haplo-insufficient manner (22), and USP20 stabilized Rad17 in a proteasome-dependent manner, USP20 itself was considered as a novel tumor suppressor protein (13). Additionally, USP20 could be ubiquitinated in a pVHL-dependent manner, inducing itself rapid degradation (23). To the best of our knowledge, the present study is the first to explore the expression and biological function of USP20 in GC.

In the present study, we evaluated the expression and clinical relevance of USP20 in GC specimens, and then we investigated the role of USP20 in GC by using silencing of USP20 expression models and USP20 overexpression model. Analyzing the USP20 and Claspin expression in TMAs obtained from one cohort of 89 cases using IHC, we found that USP20 and/or Claspin expression in GC tissues was positively correlated with the patient OS. Moreover, there was a negative correlation between USP20 expression and tumor size, tumor invasion and TNM staging. Therefore, our results revealed that USP20 is a possible tumor suppressor in GC. In line with the clinical outcomes, we found that the overexpression of USP20 could, on one hand, suppress cell proliferation and delay G1-S cell cycle transition, and on the other hand, enhance autophagy in GC cells. Furthermore, we reconfirmed that USP20 could regulate the expression of Claspin via stabilizing Claspin apart from degradation.

Claspin was found as a critical mediator in the intra-S-phase cell cycle checkpoint, which is responsible for ATR-dependent activation of Chk1 (17). Although Claspin worked as a safeguard for regulating initiation of DNA replication in normal

cells, this protective role, in terms of cancer cells, might be overridden, which would lead to their unregulated growth (24), just as DNA synthesis would be suppressed when the intra-S-phase checkpoint is activated (17). Prior studies have demonstrated that USP20 regulate the cell proliferation through the ATK-Claspin-CHK signaling pathway, and downregulation of USP20, could reduce the expression of Claspin through the ubiquitin-proteasome degradation and promote the DNA synthesis, suggesting a defect in the intra-S-phase checkpoint (15). As is known, the DNA replication occur during the S phase of cell cycle, therefore, the DNA synthesis caused by silencing of USP20 could provide a plausible explanation for the G1-S cell cycle transition acceleration which was also induced by the siUSP20.

Our result found that silencing of USP20 could inhibit autophagy, not singly but in pairs, the overexpression of USP33, which is the homology of USP20, could deubiquitinate RALB at Lys 47, and then regulate assembly of the RALB-EXO84-beclin1 complex that initiated autophagy (25). USP20 and USP33 shared 59% identity in their amino acid sequence, which may illustrate their close functional relationship (21). On the contrary, silencing of USP36 triggered autophagy via a p62-dependent pathway (18). Besides, USP19 and USP24 were also two negative regulators of autophagy flux under normal nutritional conditions (26). As a prior study reported, selective autophagy, a pathway choreographed by specific protein interactions, was directed by ubiquitin and ubiquitin-binding domains, and USP20 was likely to have unknown functions in deubiquitinating components or interactors of autophagy-associated proteins apart from its roles of deubiquitinating receptor cargo (27). The mechanisms by which USP20 positively regulate autophagy remain to be elucidated in the future study. As the above study revealed, USP36 promoted the cell proliferation and inhibited autophagy, while inhibiting autophagy have no impact on the cell proliferation (18). Thus, whether the autophagy caused by USP20 could contribute to the inhibition of tumor growth is worthy of investigation in a further study.

It should be noted that this study had limitations. A major drawback of the present study was its retrospective nature. We ignored the subsequent treatments for patients who suffered from GC recurrence, such as chemotherapy, radiotherapy and targeted therapy, which might have an influence on patients' OS outcome. Besides, we found only indirect evidence of the relationship between USP20 and Claspin in GC cells, direct evidence of the above relationship should be elucidated in a further study, such as confirming the interaction using the Co-Immunoprecipitation (Co-IP) assay.

In conclusion, our findings revealed significant correlation of USP20 in the patient outcome and the antitumor activity of USP20 by mechanisms including positive regulation of the stabilization of Claspin in GC, which render USP20 as a promising new molecular target for the design of novel therapeutic modalities to control GC development and progression.

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References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A: Global cancer statistics, 2012. *CA Cancer J Clin* 65: 87-108, 2015.
2. Chen W, Zheng R, Zeng H and Zhang S: The incidence and mortality of major cancers in China, 2012. *Chin J Cancer* 35: 73, 2016.
3. Wang S and Yuan L: Predictive biomarkers for targeted and cytotoxic agents in gastric cancer for personalized medicine. *Biosci Trends* 10: 171-180, 2016.
4. McClurg UL and Robson CN: Deubiquitinating enzymes as oncotargets. *Oncotarget* 6: 9657-9668, 2015.
5. Park JJ, Yun JH and Baek KH: Polyclonal and monoclonal antibodies specific for ubiquitin-specific protease 20. *Monoclon Antib Immunodiagn Immunother* 32: 193-199, 2013.
6. Nijman SM, Luna-Vargas MP, Velds A, Brummelkamp TR, Dirac AM, Sixma TK and Bernards R: A genomic and functional inventory of deubiquitinating enzymes. *Cell* 123: 773-786, 2005.
7. Fraile JM, Quesada V, Rodríguez D, Freije JM and López-Otín C: Deubiquitinases in cancer: New functions and therapeutic options. *Oncogene* 31: 2373-2388, 2012.
8. Qu Q, Mao Y, Xiao G, Fei X, Wang J, Zhang Y, Liu J, Cheng G, Chen X, Wang J, *et al*: USP2 promotes cell migration and invasion in triple negative breast cancer cell lines. *Tumour Biol* 36: 5415-5423, 2015.
9. Cai JB, Shi GM, Dong ZR, Ke AW, Ma HH, Gao Q, Shen ZZ, Huang XY, Chen H, Yu DD, *et al*: Ubiquitin-specific protease 7 accelerates p14(ARF) degradation by deubiquitinating thyroid hormone receptor-interacting protein 12 and promotes hepatocellular carcinoma progression. *Hepatology* 61: 1603-1614, 2015.
10. Huang Z, Wen P, Kong R, Cheng H, Zhang B, Quan C, Bian Z, Chen M, Zhang Z, Chen X, Du X, *et al*: USP33 mediates Slit-Robo signaling in inhibiting colorectal cancer cell migration. *Int J Cancer* 136: 1792-1802, 2015.
11. Kim SR, Kim JO, Lim KH, Yun JH, Han I and Baek KH: Regulation of pyruvate kinase isozyme M2 is mediated by the ubiquitin-specific protease 20. *Int J Oncol* 46: 2116-2124, 2015.
12. Li Z, Wang D, Messing EM and Wu G: VHL protein-interacting deubiquitinating enzyme 2 deubiquitinates and stabilizes HIF-1 α . *EMBO Rep* 6: 373-378, 2005.
13. Shanmugam I, Abbas M, Ayoub F, Mirabal S, Bsaili M, Caulder EK, Weinstock DM, Tomkinson AE, Hromas R and Shaheen M: Ubiquitin-specific peptidase 20 regulates Rad17 stability, checkpoint kinase 1 phosphorylation and DNA repair by homologous recombination. *J Biol Chem* 289: 22739-22748, 2014.
14. Yasunaga J, Lin FC, Lu X and Jeang KT: Ubiquitin-specific peptidase 20 targets TRAF6 and human T cell leukemia virus type 1 tax to negatively regulate NF- κ B signaling. *J Virol* 85: 6212-6219, 2011.
15. Zhu M, Zhao H, Liao J and Xu X: HERC2/USP20 coordinates CHK1 activation by modulating CLASPIN stability. *Nucleic Acids Res* 42: 13074-13081, 2014.
16. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
17. Yuan J, Luo K, Deng M, Li Y, Yin P, Gao B, Fang Y, Wu P, Liu T and Lou Z: HERC2-USP20 axis regulates DNA damage checkpoint through Claspin. *Nucleic Acids Res* 42: 13110-13121, 2014.
18. Taillebourg E, Gregoire I, Viargues P, Jacomin AC, Thevenon D, Faure M and Fauvarque MO: The deubiquitinating enzyme USP36 controls selective autophagy activation by ubiquitinated proteins. *Autophagy* 8: 767-779, 2012.
19. Gossage L, Eisen T and Maher ER: VHL, the story of a tumour suppressor gene. *Nat Rev Cancer* 15: 55-64, 2015.

20. Kim RN, Kim A, Kim DW, Choi SH, Kim DS, Nam SH, Kang A, Kim MY, Park KH, Yoon BH, *et al*: Analysis of indel variations in the human disease-associated genes CDKN2AIP, WDR66, USP20 and OR7C2 in a Korean population. *J Genet* 91: e1-e11, 2012.
21. Berthouze M, Venkataramanan V, Li Y and Shenoy SK: The deubiquitinases USP33 and USP20 coordinate beta2 adrenergic receptor recycling and resensitization. *EMBO J* 28: 1684-1696, 2009.
22. Bric A, Miething C, Bialucha CU, Scuoppo C, Zender L, Krasnitz A, Xuan Z, Zuber J, Wigler M, Hicks J, *et al*: Functional identification of tumor-suppressor genes through an in vivo RNA interference screen in a mouse lymphoma model. *Cancer Cell* 16: 324-335, 2009.
23. Li Z, Wang D, Na X, Schoen SR, Messing EM and Wu G: Identification of a deubiquitinating enzyme subfamily as substrates of the von Hippel-Lindau tumor suppressor. *Biochem Biophys Res Commun* 294: 700-709, 2002.
24. Yang CC, Suzuki M, Yamakawa S, Uno S, Ishii A, Yamazaki S, Fukatsu R, Fujisawa R, Sakimura K, Tsurimoto T, *et al*: Claspin recruits Cdc7 kinase for initiation of DNA replication in human cells. *Nat Commun* 7: 12135, 2016.
25. Simicek M, Lievens S, Laga M, Guzenko D, Aushev VN, Kalev P, Baietti MF, Strelkov SV, Gevaert K, Tavernier J, *et al*: The deubiquitylase USP33 discriminates between RALB functions in autophagy and innate immune response. *Nat Cell Biol* 15: 1220-1230, 2013.
26. Lipinski MM, Hoffman G, Ng A, Zhou W, Py BF, Hsu E, Liu X, Eisenberg J, Liu J, Blenis J, *et al*: A genome-wide siRNA screen reveals multiple mTORC1 independent signaling pathways regulating autophagy under normal nutritional conditions. *Dev Cell* 18: 1041-1052, 2010.
27. Kommaddi RP, Jean-Charles PY and Shenoy SK: Phosphorylation of the deubiquitinase USP20 by protein kinase A regulates post-endocytic trafficking of β 2 adrenergic receptors to autophagosomes during physiological stress. *J Biol Chem* 290: 8888-8903, 2015.