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ORIGINAL ARTICLE

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Ubiquitin-specific protease 3 overexpression promotes gastric carcinogenesis and is predictive of poor patient prognosis

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Although gastric cancer (GC) is one of the most common cancers, knowledge of its development and carcinogenesis is limited. To date, expression of ubiquitin-specific protease 3 (USP3) in all types of cancer, including GC, is still unknown. The present study explored the involvement of USP3 in the carcinogenesis and prognosis of GC. We measured USP3 expression in normal and GC tissues and cell lines. Correlations between USP3 protein level and clinicopathological parameters, as well as the significance of USP3 protein level for disease-free survival were assessed. Small hairpin RNA technology and transfection were used to investigate the effect of USP3 manipulation on cell proliferation and spreading. Moreover, xenograft proliferation and metastasis were used to explore the influence of USP3 on tumor growth and metastasis in animals. An increase in USP3 expression was observed in GC cells and tissues. The overexpression of USP3 was significantly correlated with several clinicopathological parameters and poor disease-free survival. Multivariate Cox regression analysis showed that the overexpression of USP3 was an independent prognostic biomarker. Silencing of USP3 suppressed GC cell proliferation and spreading in vitro as well as xenograft proliferation and metastasis in vivo; however, opposite results were obtained when USP3 was overexpressed. Further studies showed that USP3 influenced cell proliferation and spreading by regulating the cell cycle control- and

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epithelial-mesenchymal transition-related molecules. This study suggests that USP3 overexpression can be a useful biomarker for predicting the outcomes of GC patients and that USP3 targeting represents a potential modality for treating GC.

KEYWORDS
epithelial-mesenchymal transition, gastric cancer, immunohistochemistry, prognosis, ubiquitin-specific protease 3

1 | INTRODUCTION

The mortality of gastric cancer (GC) remains the third highest among all cancers.[1] According to a government report issued in 2014, the incidence of GC ranked seventh among all types of cancer in Taiwan, with more than 2000 people dying of it annually. Chemotherapy and surgical techniques do not satisfactorily enhance the survival rate of GC patients as a result of limited understanding of the pathogenesis of GC and the lack of specific targeted gene therapy.[2-4] Some studies have indicated that dysregulation of protein and RNA expression may play a crucial role in the development of GC.[5,6] Several molecules that can be used as prognostic biomarkers for GC include EPAC1, MFN2, CDKL2, CerS6, BMI1, Mel-18, miR-429 and miR-1225-5p.[7-13]

The ubiquitin-proteasome system has many critical regulatory roles in eukaryotic cellular processes, including cell cycle progression, stress response, and signal transduction.[14-16] Protein ubiquitination is both dynamic and reversible. Proteins destined for degradation are tagged with ubiquitin by ubiquitinating enzymes. Reversal of the ubiquitin conjugation of proteins relies on deubiquitinating enzymes, which cleave ubiquitin from proteins. Processes that can be regulated by deubiquitination include the rescue of proteins that are destined for degradation, the cleavage to release mature ubiquitin as well as the removal of ubiquitin to terminate or alter a biological event.[17] Currently, the five known deubiquitinating enzyme families include ubiquitin-specific proteases (USP) and ubiquitin C-terminal hydrolases.[17,18] The USP family, including USP3, is the largest family of deubiquitinating enzymes with about 60 proteases, their sizes ranging from 50 kDa to 300 kDa and sequence conservation among these proteases is limited to the catalytic domain. As the non-catalytic domains are highly diverse at the amino acid sequence level, it is hypothesized that they are important for conferring substrate specificity: for example, p53 for USP3 and USP7, and sirtuin 1 for USP22.[19-21]

As a result of different genetic contexts in various types of cells and tissues, deubiquitinating enzymes can serve as oncoproteins in certain tumors and as tumor suppressors in others.[22] Data provided by Zhang et al[23] indicated that overexpression of USP28 in lung cancer and silencing of USP28 expression in A549 lung cancer cells inhibited cell proliferation and induced apoptosis. In contrast, Liu et al[24] found that USP35 was downregulated in breast and lung cancer. Ectopic expression of USP35 in H1299 lung cancer cells inhibited cell proliferation. Previous studies have suggested that the expression of several deubiquitinating enzymes are dysregulated in GC. For instance, according to Luise et al[22] and Zeng et al,[25] USP1 and USP10 are downregulated in GC tissues and cells compared to their nontumor counterparts. Yu et al[26] also showed that UCHL1 expression was reduced in GC cell lines. To date, the expression of USP3 in various cancers, including GC, is still unknown.

Research related to the prognostic value of USP is scarce. According to the study carried out by Zhao et al,[27] overexpression of USP7 can predict a poor prognosis in lung cancer, whereas a study by Zhang et al[23] reported that the overexpression of USP28 was also associated with a poor prognosis in lung cancer patients. Furthermore, it has been found that USP2 overexpression may be a poor prognostic indicator for triple negative breast cancer.[28] To the best of our knowledge, the prognostic significance of USP3 and the role played by USP3 in GC have not yet been addressed.

The objective of the present study was to examine the expression of USP3 in normal and GC tissues and cell lines, to evaluate the possibility of using USP3 as a prognostic biomarker for GC, and to study the role of USP3 in GC tumorigenesis.

2 | MATERIALS AND METHODS

2.1 | Cell culture

We used one normal human gastric cell line (Hs738.St/Int) and seven GC cell lines (AGS, NCI-N87, TMC-1, TSGH 9201, SK-GT-2, HGC-27, and 23132/87) in this study. Sources, authentication, and maintenance of all of the cell lines are described in detail in Appendix S1.

2.2 | Study subjects

Specimens of gastric tissues from 147 consecutive patients who underwent surgical resection of GC at Taipei Medical University Wan Fang Hospital from 1998 to 2011 were retrospectively studied. Follow-up information is shown in Table 1. Clinicopathologic parameters of GC were determined based on the American Joint Committee on Cancer classification. Clinical outcome endpoint was disease-free survival. Follow-up duration for disease-free survival was defined as the period between the operation date and the date of relapse. The institutional review board of the hospital approved this study (approval no. 99049), and written informed consent was obtained from all of the patients before this study began. All

TABLE 1 Demographic data and survival of patients in different stages of GC according to the American Joint Committee on Cancer classification

	Stage I (n = 26)	Stage II (n = 40)	Stage III (n = 62)	Stage IV (n = 19)	Total (n = 147)
Gender					
Male	15	25	43	12	95
Female	11	15	19	7	52
Age (y)[a]	68.4 (11.4)	74.6 (12.1)	69.3 (13.3)	58.4 (14.7)	69.2 (13.6)
Follow-up period (d)[a]	1508.5 (1142.0)	1079.3 (841.8)	819.3 (797.6)	308.7 (220.7)	946.0 (897.4)
Survival					
Yes	23	27	20	2	72
No	3	13	42	17	75

GC, gastric cancer.
aAge and follow-up period are expressed as the mean (standard deviation).

procedures were carried out in accordance with the provisions of the Declaration of Helsinki.

2.3 | RNA extraction, RT-PCR, quantitative PCR, antibodies, protein extraction, immunoblotting, shRNA treatment, and transfection

RNA extraction, RT-PCR, quantitative PCR, antibodies, protein extraction, immunoblotting, shRNA treatment, and transfection are described in Appendix S1.

2.4 | Immunohistochemical analysis

Immunohistochemistry was used to analyze USP3 and Ki67 protein expression, as described in detail in Appendix S1.

2.5 | Colony formation assay, flow cytometric analysis, wound healing assay, in vitro invasion assay, and gelatin zymography assay

Detailed descriptions of the colony formation assay, flow cytometric analysis, wound healing assay, in vitro invasion assay, and gelatin zymography assay can be found in Appendix S1.

2.6 | Animals and tumor cell inoculation for xenograft proliferation and metastasis

Animals and tumor cell inoculation for xenograft proliferation and metastasis are described in Appendix S1. All experiments were conducted in accordance with the guidelines of the Chi Mei Medical Center Animal Ethics Research Board (approval no. 104122409).

2.7 | Statistical analysis

All of the data were analyzed using SPSS software version 24.0 (IBM, Armonk, NY, USA). All of the statistical tests were two-sided, and a P value <.05 was considered to be significant. All of the statistical analyses are described in detail in Appendix S1.

3 | RESULTS

3.1 | Ubiquitin-specific protease 3 was upregulated in GC

We first examined the expression of USP3 in the gastric cell lines to investigate the potential significance of USP3 in the development and progression of GC. RT-PCR analysis indicated that USP3 mRNA was ubiquitously expressed at higher levels in seven human GC cell lines than in the normal human gastric cell line Hs738.St/Int (Figure 1A). In parallel, as shown in Figure 1A, immunoblotting also showed that USP3 protein expression was markedly increased in all of the seven GC cell lines as compared with the Hs738.St/Int cells. Furthermore, the expressions of USP3 mRNA and protein in the tumor tissues were higher than those in the nontumor tissues as detected by RT-PCR and immunostaining (Figure 1B). An independent cohort comprising 147 GC patients was enrolled to validate the results from the RT-PCR and immunoblotting. Immunohistochemical analysis showed that USP3 was expressed at higher levels in the tumor tissues than in the nontumor tissues (Figure 1C-E). These data strongly suggested that USP3 expression is markedly elevated in GC.

3.2 | Ubiquitin-specific protease 3 upregulation correlated with GC clinicopathological characteristics and survival of GC patients

The observed upregulated expression of USP3 in GC prompted us to further investigate the clinical relevance of USP3 in the progression of GC. As shown in Table 2, the level of USP3 expression was closely correlated with Lauren classification (P = .0071), depth of invasion (P = .0061), nodal status (P = .0070), distant metastasis (P < .0001), stage (P < .0001), degree of differentiation (P = .0030), and vascular invasion (P = .0011). Representative images of USP3 expression and scores for the different parameters are shown in Figure 1F.

Furthermore, Kaplan-Meier analysis using the log-rank test showed that inferior disease-free survival was significantly associated with USP3 overexpression (P < .001) (Figure 2A). At 5 years, 20 USP3-low patients were at risk, and the disease-free survival was

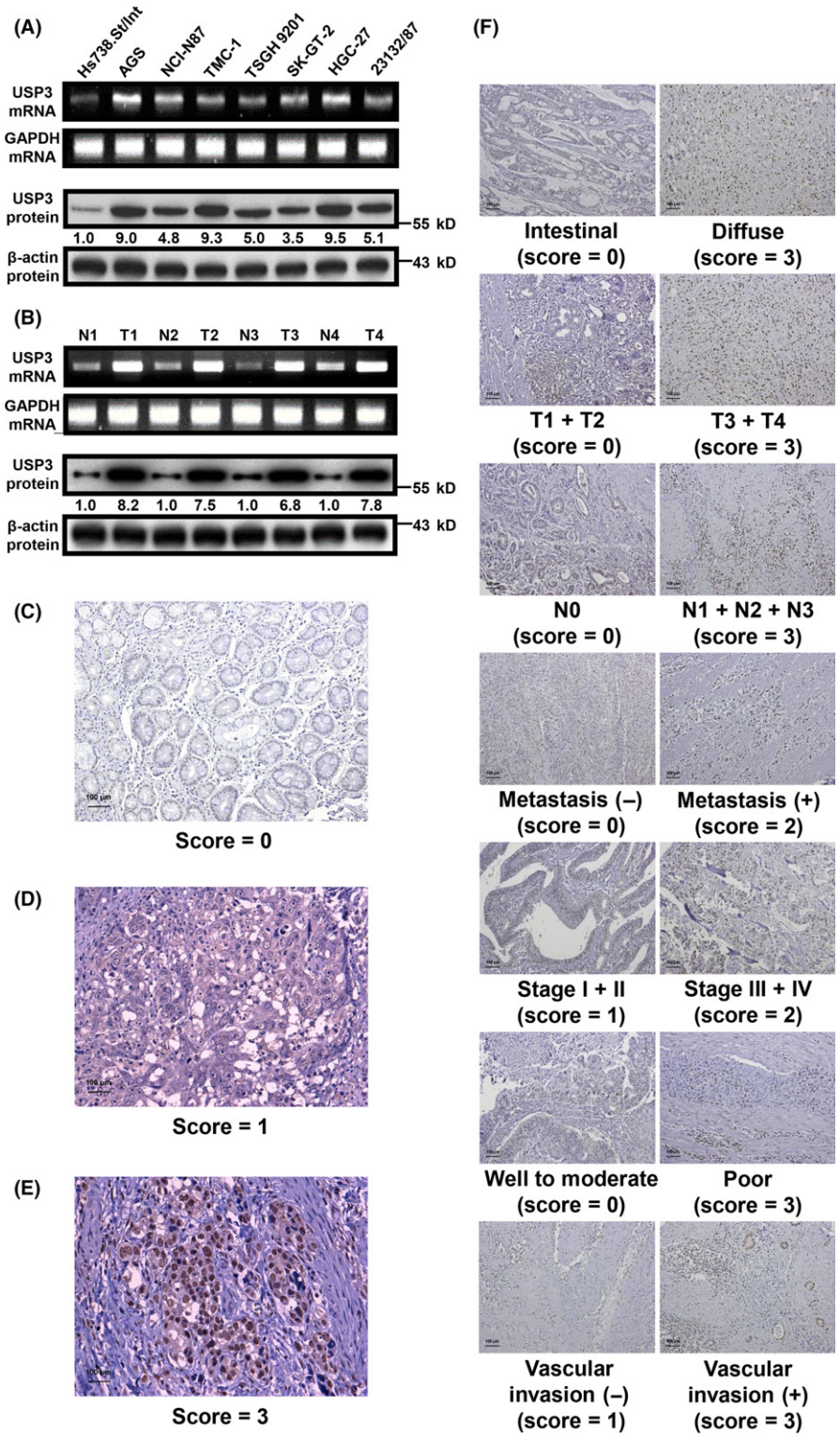

FIGURE 1 Ubiquitin-specific protease 3 (USP3) expression in gastric cell lines and tissues. A, Endogenous USP3 mRNA and protein expression was remarkably increased in gastric cancer (GC) cell lines. B, Relative changes in protein bands were measured using densitometric analysis with Hs738.St/Int cells set at 1.0-fold change as shown just below the gel data. Endogenous USP3 mRNA and protein expression was remarkably increased in the GC tissues. Relative changes in protein bands were measured using densitometric analysis with nontumor tissues set at 1.0-fold change as shown just below the gel data. N, nontumor; T, tumor. C-E, Gastric tissues analyzed by immunohistochemistry with an antibody against USP3. C, A nontumor sample without USP3 expression (score = 0). D, A tumor sample with low USP3 expression (score = 1). E, A tumor sample with high USP3 expression (score = 3). Magnification, 200×. F, Representative USP3 staining and scores for different clinicopathological parameters. Magnification, 200×. GC, gastric cancer; USP3, ubiquitin-specific protease 3

0.692 (95% confidence interval [CI], 0.578-0.806), while one USP3-high patient was at risk, and the disease-free survival was 0.221 (95% CI, 0.058-0.335).

Inferior overall survival was also significantly associated with USP3 overexpression (P <0.001) (Figure 2B). At 5 years, 20 USP3-low patients were at risk, and the disease-free survival was 0.575 (95% CI, 0.455-0.695), while two USP3-high patients were at risk, and the disease-free survival was 0.189 (95% CI, 0.069-0.309).

Table 3 summarizes the univariate analysis of the prognostic biomarkers and patient survival. USP3 overexpression (hazard ratio [HR] 0.245, 95% CI 0.142-0.426, P < .001), Lauren classification (HR 0.581, 95% CI 0.352-0.957, P = .033), depth of invasion (HR 0.210, 95% CI 0.076-0.580, P = .003), nodal status (HR 0.160, 95% CI 0.069-0.373, P < .001), distant metastasis (HR 0.054, 95% CI 0.023-0.129, P < .001), stage (HR 0.147, 95% CI 0.074-0.291, P <0.001), degree of differentiation (HR 2.093, 95% CI 1.267-3.458, P = .004),

TABLE 2 Clinical characteristics of GC patients according to high or low USP3 expression

Variable	n	USP3 expression		P*
		Score = 0 or 1 (n = 80)	Score = 2 or 3 (n = 67)	
Age (y)[a]	147	70.1 ± 13.0	68.1 ± 14.3	.3918
Gender				
Male	95	53	42	.6527
Female	52	27	25	
Lauren classification				
Intestinal	98	61	37	.0071
Diffuse	49	19	30	
Depth of invasion				
T1 + T2	30	23	7	.0061
T3 + T4	117	57	60	
Nodal status				
N0	45	32	13	.0070
N1 + N2 + N3	102	48	54	
Distant metastasis				
Absent	128	78	50	<.0001
Present	19	2	17	
Stage				
I + II	66	50	16	<.0001
III + IV	81	30	51	
Degree of differentiation				
Well to moderate	81	53	28	.0030
Poor	66	27	39	
Vascular invasion				
Absent	44	33	11	.0011
Present	103	47	56	

GC, gastric cancer; USP, ubiquitin-specific protease.
aAge is mean ± standard deviation.
*All of the statistical tests were two-sided. Significance level: $P < .05$.

and vascular invasion (HR 0.199, 95% CI 0.090-0.439, $P < .001$) were significantly correlated with disease-free survival.

In the multivariate analysis, USP3 overexpression (HR 0.501, 95% CI 0.274-0.916, $P = .025$) and distant metastasis (HR 0.101, 95% CI 0.041-0.248, $P < .001$) were prognostically independent (Table 3).

Collectively, high USP3 expression seemed to be a risk factor that predicted poor survival, suggesting that the increased expression of USP3 likely contributes to GC pathogenesis and might represent a prognostic biomarker for this disease.

3.3 | Effect of USP3 overexpression on the prognosis of advanced-stage GC

Tumor stage is an important prognostic biomarker of GC; therefore, we determined the effect of USP3 overexpression on the prognosis of early-stage (stages I and II) and advanced-stage (stages III and IV) GC. The data showed that advanced-stage GC concomitant with USP3 overexpression pointed to a significantly lower 5-year overall survival rate than advanced-stage GC without USP3 overexpression (Figure 2C, $P = .001$), whereas early-stage GC (stages I and II) was associated with a better 5-year overall survival rate regardless of the USP3 expression status (Figure 2D, $P = .371$).

3.4 | Ubiquitin-specific protease 3 accelerated GC cell proliferation and cell cycle progression

Based on the expression level of USP3, HGC-27 GC cells with a high USP3 level were chosen to help us elucidate the role of endogenous USP3 in the modulation of cell proliferation. The cells were infected with two USP3-shRNA lentiviral vectors to generate two USP3 knockdown cells (Figure 3A). As shown in Figure 3B, the ability of both cell lines to form colonies was compromised by USP3 knockdown as compared with the corresponding scrambled control cells. These results suggest that the knockdown of USP3 suppressed the ability of the GC cells to proliferate in vitro.

To dissect the biological events accompanying the alterations in cell proliferation caused by USP3, flow cytometric analysis was applied to analyze the changes in DNA content throughout the various phases of the cell cycle. As indicated in Figure 3C, USP3 knockdown HGC-27 cells showed a significant increase in the percentage of cells in the G1 phase. The percentage of USP3 knockdown cells in the G1 phase was 58.5%, whereas that of the scrambled control cells in the G1 phase was 52.1%; therefore, our shRNA experiments suggested that USP3 knockdown interferes with the G1-S transition of cell cycle progression and consequently abrogates the proliferation of GC cells. Statistical analysis with data from three independent examinations is also shown in Figure S1.

To verify the results from the HGC-27 cells, SK-GT-2 GC cells with a low USP3 level were transfected with USP3 cDNA ORF vectors and stable clones were selected (Figure 3A). As shown in Figure 3B, a colony formation assay showed that after transfection with the USP3 cDNA ORF vector, the SK-GT-2 cells formed more colonies. Overall, our overexpression experiments indicate that USP3 might function as an oncoprotein that is capable of facilitating the cell cycle, and thus able to accelerate the progression of cell proliferation.

3.5 | Ubiquitin-specific protease 3 facilitated cell cycle progression by increasing the regulators cyclins D and E

To identify the molecular mechanisms that govern USP3 knockdown-induced G1 arrest, we assessed the expression of various cyclins involved in cell cycle control in the USP3 knockdown GC cells. Reduced expression of cyclins D and E, both of which are involved in the regulation of the G1 phase, was observed in the USP3 knockdown HGC-27 GC cells (Figure 3D). In contrast, the expression of cyclins A and B, which are both involved in the regulation of the G2 phase, was slightly decreased (Figure 3D).

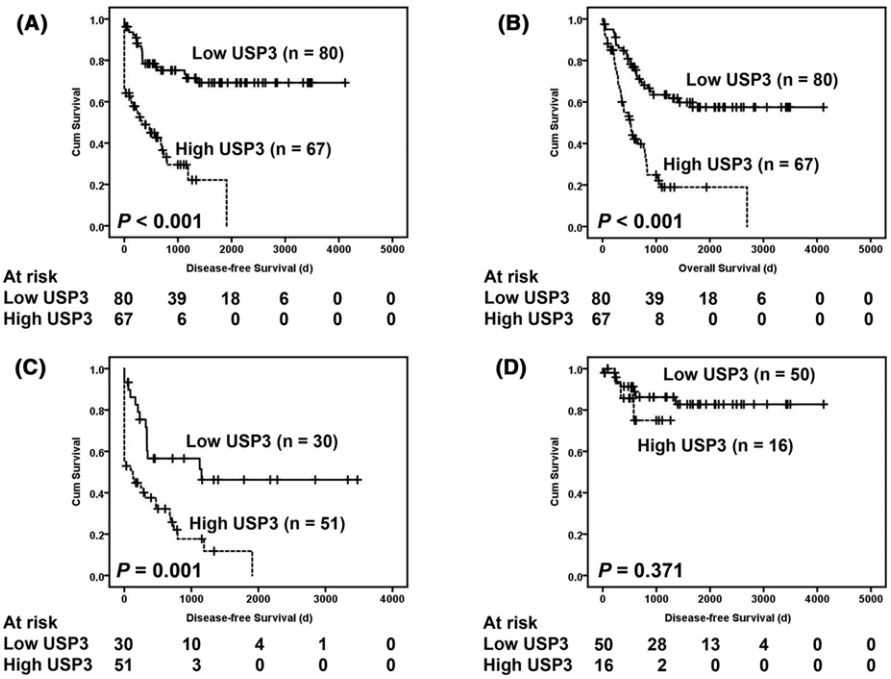

FIGURE 2 Survival analysis of gastric cancer (GC) patients stratified by ubiquitin-specific protease 3 (USP3) immunoreactivity. A, Disease-free survival. B, Overall survival. C, Disease-free survival in advanced-stage GC (stages III and IV). D, Disease-free survival in early-stage GC (stages I and II). All of the statistical tests were two-sided. Significance level: $P < .05$

TABLE 3 Univariate and multivariate Cox regression analyses of prognostic biomarkers and survival in 147 GC patients

	Univariate		Multivariate	
Variable	HR (95% CI)	P*	HR (95% CI)	P*
USP3				
Low expression vs High expression	0.245 (0.142-0.426)	<.001	0.501 (0.274-0.916)	.025
Lauren classification				
Intestinal vs Diffuse	0.581 (0.352-0.957)	.033	1.953 (0.890-4.285)	.095
Depth of invasion				
T1 + T2 vs T3 + T4	0.210 (0.076-0.580)	.003	0.779 (0.246-2.463)	.670
Nodal status				
N0 vs N1 + N2 + N3	0.160 (0.069-0.373)	<.001	0.538 (0.172-1.683)	.287
Distant metastasis				
Negative vs Positive	0.054 (0.023-0.129)	<.001	0.101 (0.041-0.248)	<.001
Stage				
I + II vs III + IV	0.147 (0.074-0.291)	<.001	0.467 (0.166-1.313)	.149
Degree of differentiation				
Well to moderate vs Poor	2.093 (1.267-3.458)	.004	1.822 (0.835-3.976)	.132
Vascular invasion				
Negative vs Positive	0.199 (0.090-0.439)	<.001	0.534 (0.221-1.290)	.163

CI, confidence interval; GC, gastric cancer; HR, hazard ratio; USP, ubiquitin-specific protease.

*All of the statistical tests were two-sided. Significance level: $P < .05$.

Furthermore, the amount of the cyclin-dependent kinases, CDK1 and CDK2, was also slightly decreased in the USP3 knockdown HGC-27 cells (Figure 3D).

Ubiquitin-specific protease 3-overexpressing SK-GT-2 cells were used to validate the results from the HGC-27 cells. Elevated expression of cyclins D and E was observed in the USP3-overexpressing SK-GT-2 cells (Figure 3D). As seen in the results from the HGC-27 cells, the expression of cyclin A and cyclin B was slightly increased (Figure 3D). Furthermore, the amount of the cyclin-dependent kinases, CDK1 and CDK2, was also slightly increased in the USP3-overexpressing SK-GT-2 cells (Figure 3D). These data also indicate that USP3 played an important role in cell cycle progression.

Quantitative PCR was carried out to quantitate the mRNA level of cyclin D in USP3-manipulated GC cells. Results indicated that the

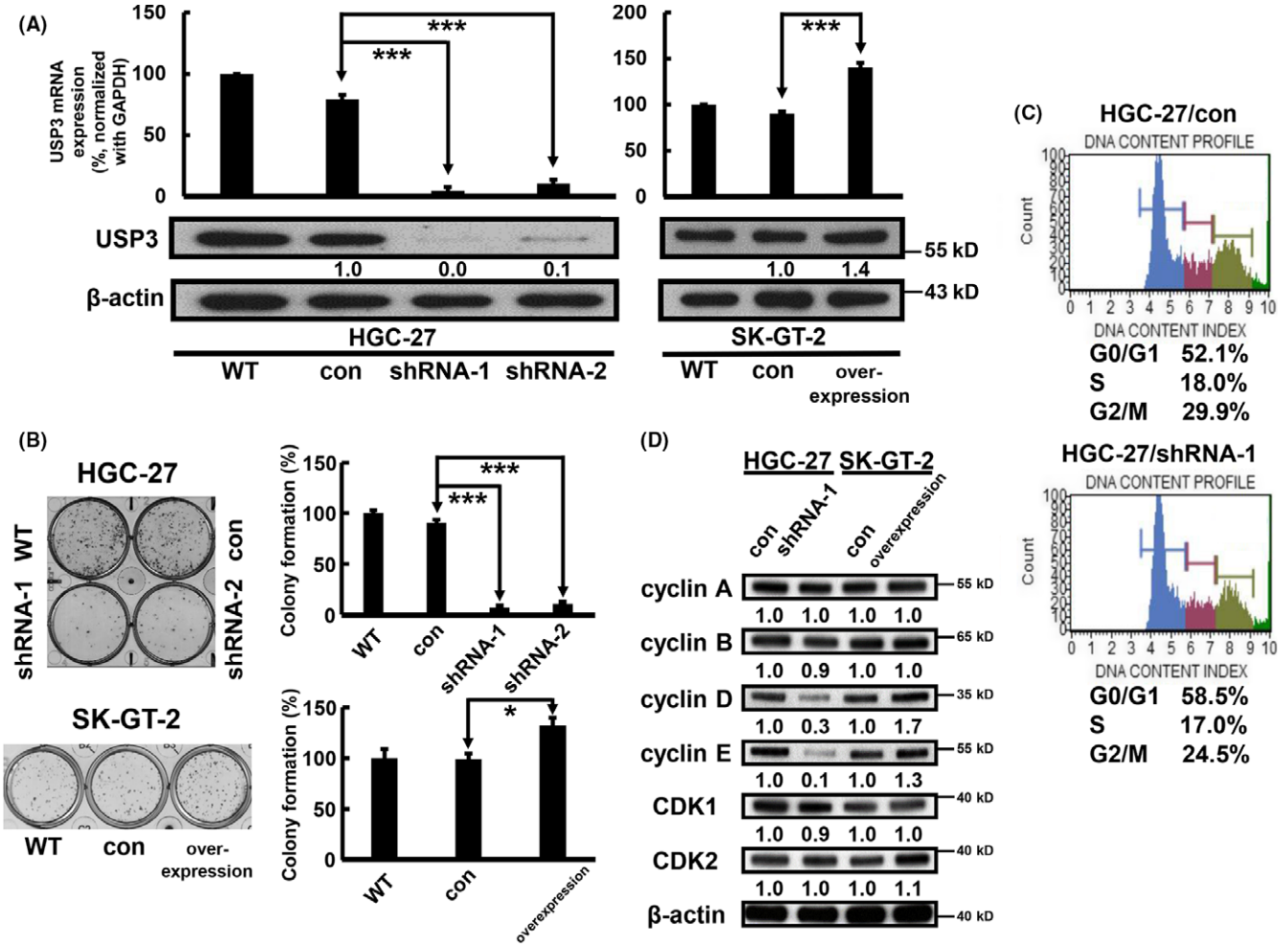

FIGURE 3 Verification of ubiquitin-specific protease 3 (USP3) manipulation in HGC-27 and SK-GT-2 cells, and the effect of stable USP3 manipulation on cell growth, cell cycle distribution, and the expression of cell cycle control molecules in the cells. A, Quantitative PCR and immunoblotting results indicate that USP3 was efficiently knocked down by shRNA treatment and overexpressed by transfection. Bar graph represents USP3 mRNA expression relative to the control group (%, presented as the mean ± standard deviation, ***$P < .001$). Relative changes in protein bands were measured using densitometric analysis with WT HGC-27 and SK-GT-2 cells set at 1.0-fold change as shown just below the gel data. B, Stable USP3 knockdown and overexpression resulted in significantly decreased and increased colony formation, respectively. The photomicrographs shown are from one representative experiment carried out in triplicate with similar results. Bar graph represents the colony numbers relative to the control group (%, presented as the mean ± standard deviation, *$P < .05$, ***$P < .001$). C, Stable USP3 knockdown resulted in a sustained accumulation of cells in the G1 phase. Cellular distribution (as %) in the different phases of the cell cycle (G0/G1, S, and G2/M) is presented. D, Stable USP3 knockdown and overexpression obviously decreased and increased the expression of the cell cycle control molecules, respectively. The typical result from three independent experiments is shown. Relative changes in protein bands were measured using densitometric analysis with scrambled control HGC-27 cells and SK-GT-2 cells transfected with empty vectors set at 1.0-fold change as shown just below the gel data

mRNA levels of cyclin D were correlated with their protein levels in USP3-manipulated GC cells (Figure S2). The results suggest that the protein levels of cell cycle control-related molecules are not affected by USP3-mediated deubiquitination, that is, cell cycle control-related molecules are not the direct substrates of USP3.

3.6 | Ubiquitin-specific protease 3 sped up the migration and invasion of GC cells

To verify the effect of USP3 knockdown on the migration of GC cells, we carried out a wound healing assay and observed a significant delay in wound closure in the USP3 knockdown HGC-27 cells compared with the scrambled control cells (Figure 4A). In the cell invasion assay, USP3 knockdown significantly suppressed cell invasion compared with the scrambled control (Figure 4A).

Matrix metalloproteinase-2 (MMP-2) is involved in the breakdown of the extracellular matrix in disease processes, such as metastasis.[29] It is considered that the tissue degradation following plasminogen activation facilitates tissue invasion, and thus contributes to metastasis. We used gelatin zymography to analyze the effects of USP3 knockdown on the enzymatic activity of MMP-2. As indicated in Figure 4C, USP3 knockdown clearly inhibited the

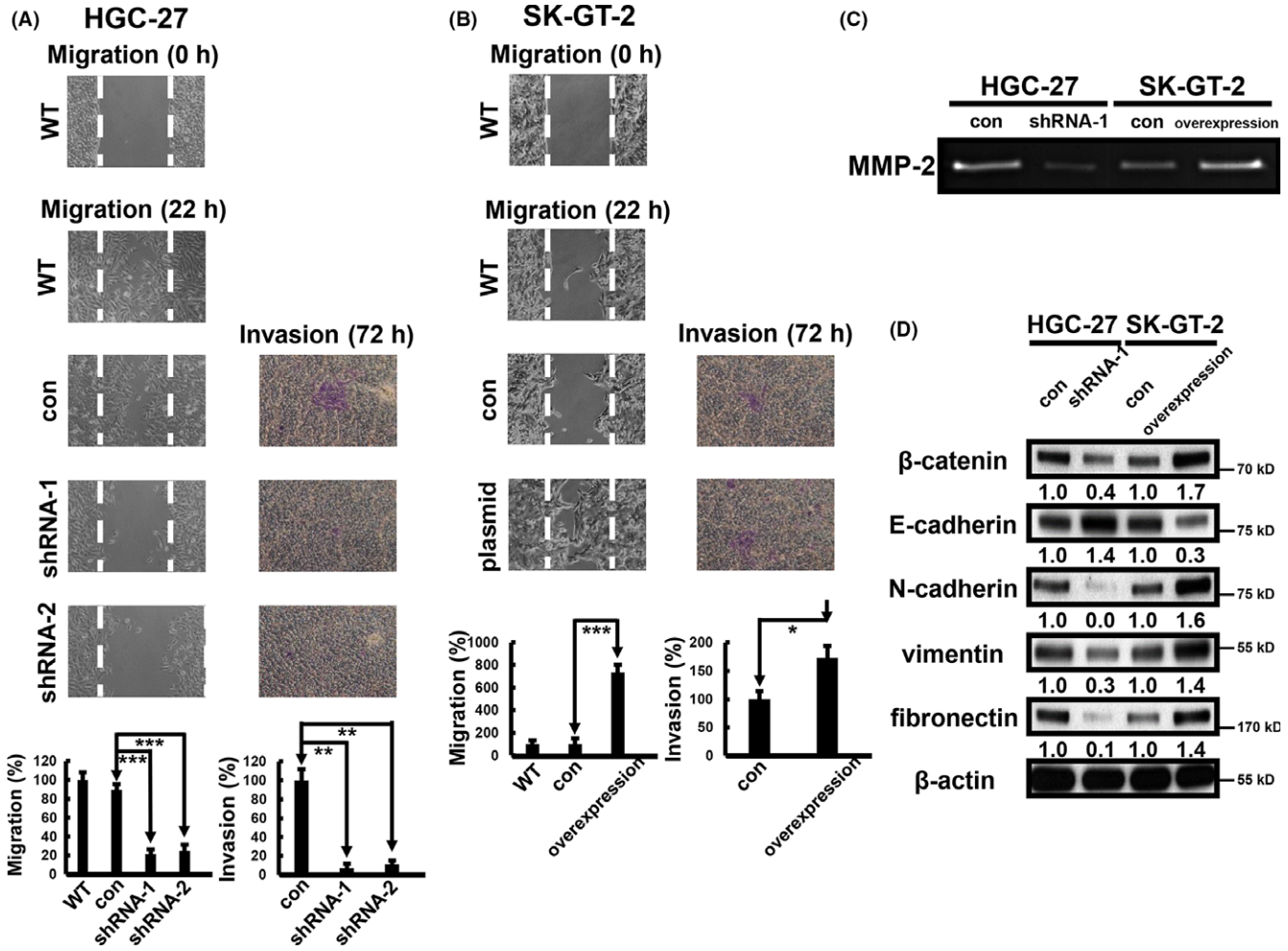

FIGURE 4 Effect of ubiquitin-specific protease 3 (USP3) manipulation in HGC-27 and SK-GT-2 cells on cell spreading and the activity and expression of metastasis-related molecules. A, Stable USP3 knockdown significantly decreased HGC-27 cell migration and invasion. The photomicrographs shown are from one representative experiment carried out three times with similar results. Bar graph represents the number of migrated and invaded cells relative to the control group (%, presented as the mean ± standard deviation, $^{**}P < .01$, $^{***}P < .001$). B, Stable USP3 overexpression significantly increased SK-GT-2 cell migration and invasion. The photomicrographs shown are from one representative experiment carried out three times with similar results. Bar graph represents the number of migrated and invaded cells relative to the control group (%, presented as the mean ± standard deviation, $^{*}P < .05$, $^{***}P < .001$). C, Stable USP3 overexpression and knockdown markedly increased and decreased the activity of MMP-2. The typical result from three independent assays is shown. D, Stable USP3 manipulation resulted in dysregulated expression of epithelial-mesenchymal transition (EMT)-related molecules. A typical result from three independent experiments is shown. Relative changes in protein bands were measured using densitometric analysis with scrambled control HGC-27 cells and SK-GT-2 cells transfected with empty vectors set at 1.0-fold change as shown just below the gel data

activity of MMP-2 compared with the scrambled control. These results suggest that USP3 knockdown suppresses cell spreading by inhibiting the activity of MMP-2.

The role of USP3 in cell migration led us to examine whether USP3 had any effect on epithelial-mesenchymal transition (EMT) in the GC cells. Immunoblotting results showed that USP3 knockdown induced the expression of the epithelial cell marker (E-cadherin) and reduced the expression of the mesenchymal cell markers (β-catenin, N-cadherin, vimentin and fibronectin), in the HGC-27 cells (Figure 4D). These data suggest that USP3 regulates the EMT process, resulting in enhanced migratory ability of GC cells in vitro.

Ubiquitin-specific protease 3-overexpressing SK-GT-2 cells were used to confirm the results from the HGC-27 cells. By supporting the role of USP3 in promoting cell motility, USP3 overexpression in the SK-GT-2 cells increased the migration, invasion, and activity of MMP-2 (Figure 4B,C). Further, USP3 overexpression upregulated the expression of β-catenin, N-cadherin, vimentin, and fibronectin, and it downregulated the expression of E-cadherin (Figure 4D).

Quantitative PCR was carried out to quantitate the mRNA level of N-cadherin in USP3-manipulated GC cells. Results indicated that the mRNA levels of N-cadherin were correlated with their protein levels in USP3-manipulated GC cells (Figure S3). The results suggest that the protein levels of EMT-related molecules are not affected by

USP3-mediated deubiquitination, that is, EMT-related molecules are not the direct substrates of USP3.

3.7 | Ubiquitin-specific protease 3 promoted GC tumor growth and metastasis in vivo

The in vitro data that USP3 positively modulated the GC cell cycle and proliferation prompted us to ask whether USP3 can promote GC growth and metastasis in vivo. In this study, each experimental mouse bearing USP3 knockdown or scrambled control HGC-27 cells on the right hind flank began to show conspicuous differences in tumor growth between these two groups. After 3 weeks, tumors of the control group showed a 9.1-fold higher weight than the USP3 knockdown tumors (Figure 5A). In parallel, this trend was also confirmed by the sizes of the dissected tumors (Figure 5A). Figure S4A shows that USP3 knockdown significantly decreased the mitotic index, shown as the expression of Ki67, compared with the control group. This result suggested that the increased tumor volume was due to the accelerated proliferation. Expression of USP3 in the xenograft was observed by immunohistochemistry (Figure 5B). All of the animals appeared healthy, with no loss of body weight noted during the experiments (Figure S5).

In contrast, the mice bearing USP3-overexpressing or vector control SK-GT-2 cells were used to validate the results from the HGC-27 cells. The USP3-overexpressing tumors showed a 9.4-fold higher weight than the vector control tumors (Figure 5C). Sizes of the dissected tumors also confirmed this trend (Figure 5C). Figure S4B shows that USP3 overexpression significantly increased the mitotic index compared with the control group. Collectively, these data strongly suggest that USP3 markedly accelerated the proliferation of the tumor cells.

Finally, we wanted to know whether USP3 knockdown also influenced the metastatic potential of HGC-27 cells in vivo. As shown in Figure 5D, the mice in the control group showed disseminated tumors in the mesentery and abdominal cavity at 6 weeks after i.p. injection. However, fewer and smaller tumors were observed in the mice in the USP3 knockdown group. Expression of USP3 in the metastatic tumors was also observed (Figure 5E). Taken together, these results suggest that USP3 clearly enhanced GC metastasis in vivo.

4 | DISCUSSION

The important role deubiquitinating enzymes play in cell proliferation indicates an association between these enzymes and tumorigenesis. Accumulated data have shown that deubiquitinating enzymes are significantly dysregulated in many types of cancer. For example, the study carried out by McFarlane et al[30] identified high expression of USP17 in lung, colon, esophagus and cervix cancer. Luise et al[22] reported the first comprehensive screening of deubiquitinating enzyme dysregulation in different human cancers by in situ hybridization on tissue microarrays; they used a GC progression tissue microarray to show that expression of USP1 and STAMBP was significantly decreased from normal to the metastatic state. In addition, Weng et al[31] found that the expression of OTUB1 in GC tissues was higher than that in nontumor tissues. Furthermore, Gu et al[32] examined UCHL1 expression, which was higher in primary GC tissues and liver metastases from GC than in nontumor tissues. The expression of USP3 in human cancers is still unknown, and the present study is the first to investigate the expression of USP3 in gastric tissues and cells. The results of immunohistochemistry, RT-PCR, and immunoblotting showed that USP3 was overexpressed in both GC tissues and cells, and suggested an association between USP3 and GC tumorigenesis. Zhang et al[23] found that USP28 was overexpressed in lung cancer and it was a direct target gene of microRNA-4295. The elevated expression of USP28 has been found to be due in part to the reduced expression of microRNA-4295. MicroRNA-let-7a, another microRNA identified by Liu et al,[24] was shown to be a positive regulator of USP35 expression. However, the mechanism for the upregulation of USP3 in GC remains to be investigated.

Many studies have examined the role of deubiquitinating enzymes in cell proliferation. McFarlane et al[30] found that USP17 expression in HeLa cells was cell cycle regulated and silencing of USP17 expression inhibited cell proliferation and caused cell cycle arrest in the G1 phase. Data shown by Hou et al[33] also indicated that the expression of USP42 in GC tissues was higher than that in nontumor tissues. Abrogating USP42 in two GC cells inhibited cell proliferation and stimulated G1 phase arrest. In addition, the proteins promoting cell cycle progression (cyclin D1, cyclin E1, and PCNA) were downregulated in USP42 knockdown cells. One study carried out on 293T cells showed that USP3 knockdown led to delay of S phase progression.[34] In this study, we explored the role of USP3 in GC cell proliferation by manipulating the expression of USP3, and the results accord with those mentioned above. Knockdown of USP3 reduced the number of colonies, whereas overexpression of USP3 increased them, indicating that USP3 facilitated cell proliferation. Moreover, the knockdown of USP3 reduced the number of colonies because the cell cycle was arrested in the G1 phase. We also obtained consistent results in detecting the expression of cell cycle-related molecules. We found that the amount of cyclins D and E involved in the G1 phase was positively correlated with that of USP3; in addition, the amount of cyclins A and B involved in the G2 phase remained unchanged. These data were further confirmed by the in vivo xenograft proliferation assay. Collectively, these data showed that USP3 promoted the proliferation of GC cells through driving the G1-S transition. Details of how USP3 regulates cell proliferation and the expression of cell cycle-related molecules remains to be elucidated.

A high incidence of metastasis is still one of the main reasons for the poor survival of GC patients.[4] Several members of the deubiquitinating enzyme family are known to contribute to cell migration and invasion, including USP2,[28] OTBU1,[31] and USP42.[33] As shown by Zhou et al,[35] knockdown of OTUB1 expression decreased migration and invasion in colon cancer cells in association with dysregulation of EMT marker expression. We examined the role of USP3 in GC

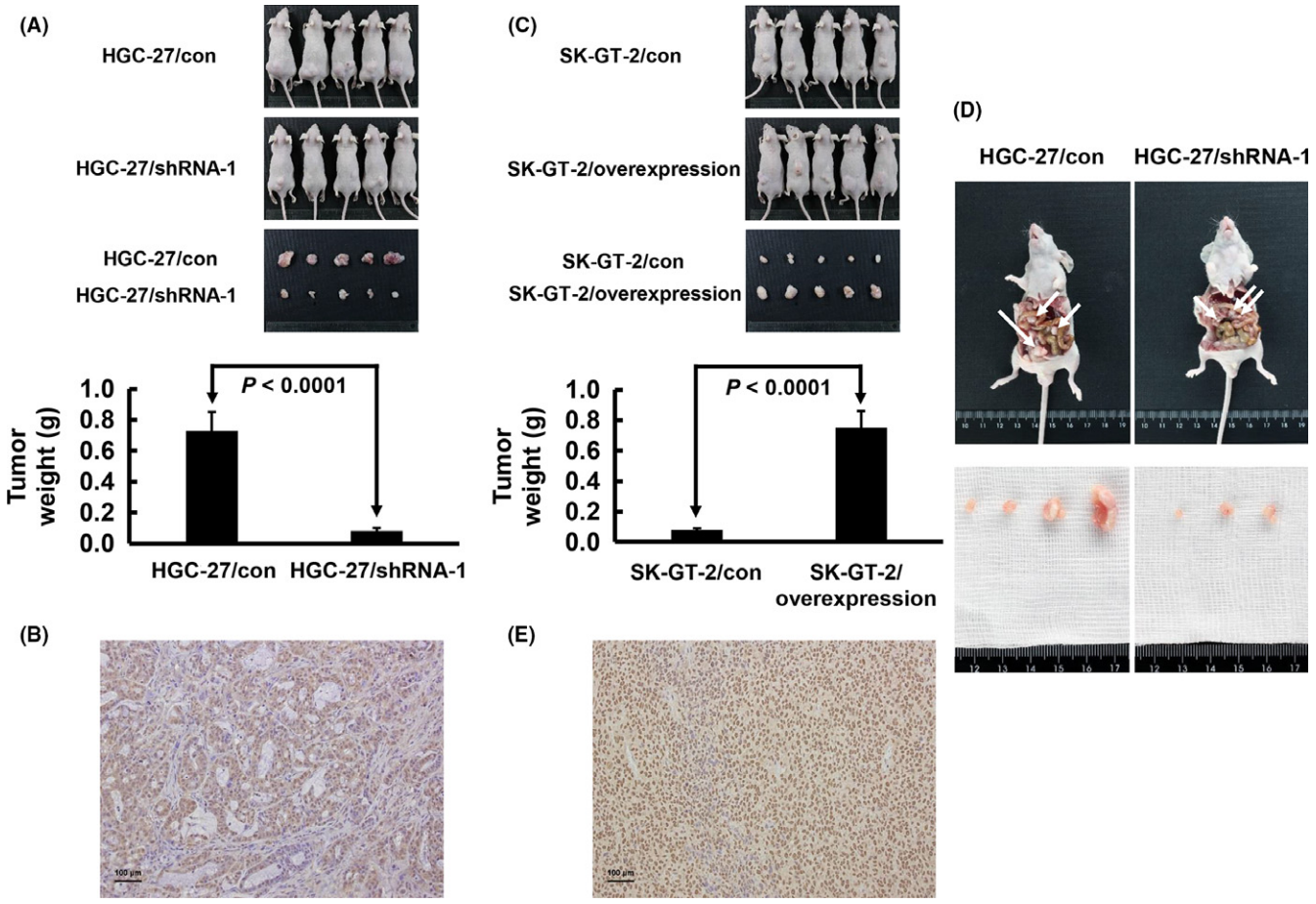

FIGURE 5 Effect of ubiquitin-specific protease 3 (USP3) manipulation in HGC-27 and SK-GT-2 cells on in vivo tumor growth and metastasis in nude mice. A, Stable USP3 knockdown resulted in the significant suppression of HGC-27 xenograft proliferation. Bar graph represents the results of the average tumor weight (presented as the mean ± standard deviation, n = 5). B, Expression of USP3 in the xenograft. Magnification, 200×. C, Stable USP3 overexpression resulted in significant enhancement of SK-GT-2 xenograft proliferation. Bar graph represents the results of the average tumor weight (presented as the mean ± standard deviation, n = 5). D, Stable USP3 knockdown resulted in the obvious suppression of HGC-27 xenograft metastasis. Peritoneal dissemination was recognized from the tumor nodules visualized in the abdominal cavity and mesenterium. Upper pictures show the peritoneal disseminations and the lower pictures show the dissected tumor nodules. Arrows in the pictures indicate the tumors developing peritoneal metastasis. E, Expression of USP3 in the metastatic tumor nodule. Magnification, 200×

cell migration and invasion because our results from the χ^2 test showed that the expression of USP3 correlated with several metastasis-related clinicopathological parameters. These results, which agree with those mentioned above, indicated that USP3 facilitated the migration and invasion of the GC cells. We also found the expression of USP3 in the GC cells to be positively correlated with the enzymatic activity of MMP-2. Given that MMP are related to tumor metastasis, we suggest that the expression of USP3 in GC cells facilitates the expression of MMP-2, resulting in cell migration and invasion. Furthermore, we observed that the levels of several EMT marker genes, including β-catenin, N-cadherin, vimentin, and fibronectin, were positively correlated with USP3 expression in the GC cells, whereas the expression of E-cadherin was dramatically increased after USP3 was knocked down. These conclusions were further supported by in vivo metastasis experiments. Taken together, these results showed that USP3 played an important role in GC cell migration and invasion through modulation of EMT. Future studies should focus on the molecules that are downstream of USP3 to illuminate the details of the regulatory network controlled by USP3 in GC.

Prognosis is critical for patients with GC, especially advanced GC. Few studies have been conducted on the relationship between deubiquitinating enzymes and prognosis. According to the statistical analysis carried out by Weng et al[31] and Zhou et al,[35] overexpression of OTUB1 can be a prognostic biomarker for gastric and colon cancer. Another study conducted by Nishimura et al[36] also showed that high USP44 expression conferred a poorer prognosis for GC. The only study conducted on colorectal cancer showed that patients with downregulated USP3 mRNA expression had a significantly poorer survival rate compared with patients without this change.[37] However, in the present study, we found that overexpression of USP3 correlated with poor disease-free survival of patients with GC,

and that USP3 could be a prognostic biomarker for GC. One reason for the discrepancy between our study and Wang's study may result from the different molecules examined. In our study, USP3 protein was measured, and in another, USP3 mRNA was detected. Another explanation for the discrepancy is that USP3 expression is cell context-specific. According to the Oncomine database, USP3 was upregulated in brain and bladder cancer, but downregulated in colorectal, leukemia and ovarian cancer. Overall, these studies suggested that the expression of USP3 in human cancers seems to be more complicated than expected and warrants additional studies. To our knowledge, the present study is the first to show that USP3 overexpression is a prognostic biomarker for GC. In addition, the current results indicated that in patients with advanced GC, USP3 correlated with poor disease-free survival; therefore, intensive follow-up programs are needed for these patients.

In the current study, we examined the expression of USP3 in normal and GC tissues and cell lines to evaluate the possibility of using USP3 as a prognostic biomarker for GC, and to study the role of USP3 in GC tumorigenesis. We found that USP3 was overexpressed in GC tissues and cells. Furthermore, USP3 overexpression can be a useful biomarker for predicting the outcomes of GC patients. Moreover, mechanistic studies further showed that USP3 influenced cell proliferation and spread by regulating cell cycle control and metastasis-related protein. In vivo experiments showed that USP3 promoted GC tumor growth and metastasis. Our findings suggest that USP3 targeting represents a potential modality for treating GC.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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