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Original article

Ubiquitin-specific protease 1 acts as an oncogene and promotes lenvatinib efficacy in hepatocellular carcinoma by stabilizing c-kit

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ABSTRACT

Introduction and Objectives: Ubiquitin-specific proteases (USPs) act as proto-oncogenes or tumor suppressors in a wide variety of cancers. In this study, we intended to explore the role of USP1 in hepatocellular carcinoma (HCC).
Materials and Methods: The clinical significance of USP1 in HCC was analyzed based on The Cancer Genome Atlas (TCGA) data and immunohistochemical staining. siRNAs and lentivirus were used to knock down and overexpress indicated genes, respectively. qRT-PCR and immunoblotting were performed to examine mRNA and protein expression, respectively. CCK8, colony formation and PI/Annexin V-APC staining were performed to examine cellular function. Immunoprecipitation, coomassie blue staining, mass spectrum and immunoblotting were conducted to evaluate the interaction between USP1 and c-kit.
Results: USP1 was over-expressed in HCC patients. Patients with high expression of USP1 had shorter overall and disease free survival than those with low expression of USP1. Functional results showed that USP1 was critical for HCC cell growth and proliferation. Immunoprecipitation and immunoblotting results suggested that USP1 interacted with c-kit and promoted the stability of c-kit, which is an important target of lenvatinib in HCC. Knockdown of c-kit reversed the oncogenic function of USP1 on HCC cell growth. Lastly, USP1 upregulation conferred higher sensitivity of HCC cells to lenvatinib treatment.
Conclusions: Our study demonstrated that USP1 acted as an oncogene in HCC. It also promoted lenvatinib efficacy by stabilizing c-kit.

1. Introduction

Hepatocellular carcinoma (HCC) is the second and sixth most common malignancy in men and women [1]. During the past decades, a wide range of proto-oncogenes are identified to play pivotal roles in HCC development. Based on these studies, multi-kinase inhibitor lenvatinib is developed to treat HCC [2,3]. Recently, immune checkpoint inhibitors (ICIs) have been demonstrated effective for the treatment of solid tumors, including melanoma [4], whereas HCC patients show less response to ICIs [5]. Combinations of ICIs and lenvatinib are thought as the future treatment of advanced HCC patients [6]. Since most of the patients are resistant to lenvatinib, understanding the molecular events triggering lenvatinib resistance would help the oncologists to formulate a better treatment strategy for this deadly malignancy.

Ubiquitin-specific proteases (USPs) are important post-transcription factors that regulate the protein stability of downstream effectors through modulating their ubiquitination [7]. Dys-regulation of USPs plays an important role in the development of various cancers, including HCC [8,9]. As a member of USPs, USP1 also participates in cancer development through regulating kinds of downstream targets. For example, USP1 stabilizes Snail to promote ovarian cancer stemness and metastasis [10]. Reducing the expression of USP1 suppresses breast cancer metastasis through degradation of KPNA2 [11]. Silencing of USP1 retards multiple myeloma malignant growth through regulating DNA repair [12]. Dys-regulation of USP1 also regulates chemotherapy sensitivity in colorectal cancer [13]. These results indicate that USP1 acts as an oncogene in various cancers. However, the

Abbreviations: USPs, ubiquitin-specific proteases; HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time PCR; PCNA, proliferating cell nuclear antigen; VEGF, vascular endothelial growth factor; FGFR, fibroblast growth factor receptors; PDGFRα, platelet-derived growth factor receptor-alpha

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function of USP1 and its connection with lenvatinib are poorly understood in HCC.

In this study, we analyzed the clinical relevance of USP1 in HCC. We also knocked down and overexpressed USP1 in HCC cells and examined the function and mechanism of USP1 in HCC. We demonstrated that overexpression of USP1 promoted HCC cell growth, proliferation and lenvatinib efficacy through stabilization of c-kit.

2. Materials and methods

2.1. TCGA database analysis of USP1

The transcript level of USP1 in HCC tissues and normal tissues was analyzed from the Cancer Genome Atlas website (http://cancergenome.nih.gov).

2.2. Cell culture

Human liver normal cells LO2 and HCC cell lines HUH7 and SKHEP1 were obtained from American Type Culture Collection (USA). These cells were grown in Dulbecco's modified Eagle's medium (DMEM) culture medium, which was supplied with 10% fetal bovine serum (Gibco, California, USA) and 1% penicillin/streptomycin solution (Corning, New York, USA). The cell culture was maintained in a 37 °C incubator with 5% CO_2.

2.3. Immunohistochemical staining

Human HCC tissue microarray was fixed in 4% paraformaldehyde. Paraffin-embedded tissues (4-5 μm) were subjected to deparaffinization and hydration in xylene and graded alcohol, respectively. After antigen repair with citrate buffer (pH = 6), 3% hydrogen peroxide was used to block endogenous peroxidase. Goat serum (10%) was used to block the slides. The tissues were stained with USP1 antibody (1:800 dilution, Proteintech).

2.4. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen). The RNA was reversely transcribed using M-MLV reverse transcriptase (Promega, Madison, Wisconsin, USA). qPCR was carried out on a Bio-Rad CFX instrument using TransStart Top Green qPCR SuperMix (TransGen Biotech, Beijing, China). The primer sequences were as follows: c-kit forward, 5'-CGTTCTGCTCCTACTGCTTCG-3', and reverse, 5'-CCCACGCGGACTATTAAGTCT-3'; GAPDH forward, 5'-TGACTTCAACAGCGACACCCA-3', and reverse, 5'-CACCCTGTTGCTGTAGCCAAA-3'. GAPDH served as the internal control.

2.5. Western blot assays

The proteins were isolated from cells using RIPA buffer (Beyotime, Shanghai, China). BCA kit was used to determine the protein concentration. The proteins were then subjected to the following experiments: 1, separation on SDS-PAGE gels; 2, transfer to PVDF membranes; 3, blockage with 5% skim milk; 4, incubation with primary antibodies and secondary antibodies; and 5, signal detection with an ECL-Plus kit. Antibodies against USP1 and GAPDH were purchased from Proteintech. Secondary antibodies were from Santa Cruz (CA, USA).

2.6. Cell proliferation analysis

Equal numbers of HUH7 and SKHEP1 cells transfected with siCtrl and siUSP1, Ctrl and USP1 overexpressing lentivirus were seeded into 96-well plates with 200 μl culture medium. After 6, 30, 54 and 78 h, 20 μl of CCK-8 reagent was added to each well, followed by incubation in a 37 °C incubator and measurement of the OD450 value under a microplate reader. The OD value at 6 h was recognized as 0 h, as shown in the figure. The cell viability at other time points was normalized to that at 6 h.

2.7. Colony formation assay

A total of 1000 HUH7 and a total of 500 SKHEP1 cells were seeded into 6-well plates. After 7-10 days, cell colonies were washed with PBS, fixed with methanol and stained with crystal violet.

2.8. Immunoprecipitation

USP1-Flag or c-Kit-Flag overexpressing vectors were transfected into HEK293 cells for 48 h. After immunoprecipitating with IgG and Flag antibody, the protein lysates, including Input, IP-IgG and IP-Flag were subjected to immunoblotting assay with indicated antibodies.

2.9. Statistical analysis

The data were shown as mean ± SEM of three independent experiments. GraphPad Prism software was used to analyze the data and statistical significance. Student's t test was used to compare the differences between two groups. One-way ANOVA was used when more than two groups were included. Statistical analysis was considered significant when $P < 0.05$.

3. Results

3.1. High expression of USP1 is associated with the poorer survival of HCC patients

We firstly analyzed the mRNA expression of USP1 from TCGA data, in which 368 tumor and 160 normal tissues were included. Comparing with normal tissues, USP1 was upregulated in HCC tissues (Fig. 1A). USP1 high expression was correlated with higher AJCC and T stage, but was not associated with the metastasis and nodal status of the patients (Table 1). Then, we performed survival analysis in HCC patients who were subdivided into USP1 high and low expression group. The patients with USP1 high expression had relatively shorter overall and disease free survival as compared with those with USP1 low expression, indicating that USP1 may be associated with HCC progression (Fig. 1B). In addition, we used IHC staining to detect USP1 protein abundance in HCC and paired normal tissues, and the results showed that USP1 was upregulated in HCC samples (Fig. 1C). USP1 high expression was also correlated with the AJCC stage but not with the grade of HCC patients (Table 2). Lastly, comparing with liver normal cells LO2, HCC cells, including HUH7 and SKHEP1, had higher mRNA expression of USP1 (Fig. 1D). Taken together, overexpression of USP1 is a potential driver for HCC development.

3.2. Upregulation of USP1 promotes HCC cell growth

Next, we examined whether USP1 exhibited oncogenic role in HCC by performing loss-of-function and gain-of-function experiments. siRNAs were used to knock down USP1 and the immunoblotting results found that USP1 was obviously down regulated in HUH7 and SKHEP1 cells after transfecting with siRNAs against USP1 (Fig. 2A). CCK8 assays were used to assess cell proliferation. Knockdown of USP1 significantly repressed the growth of both

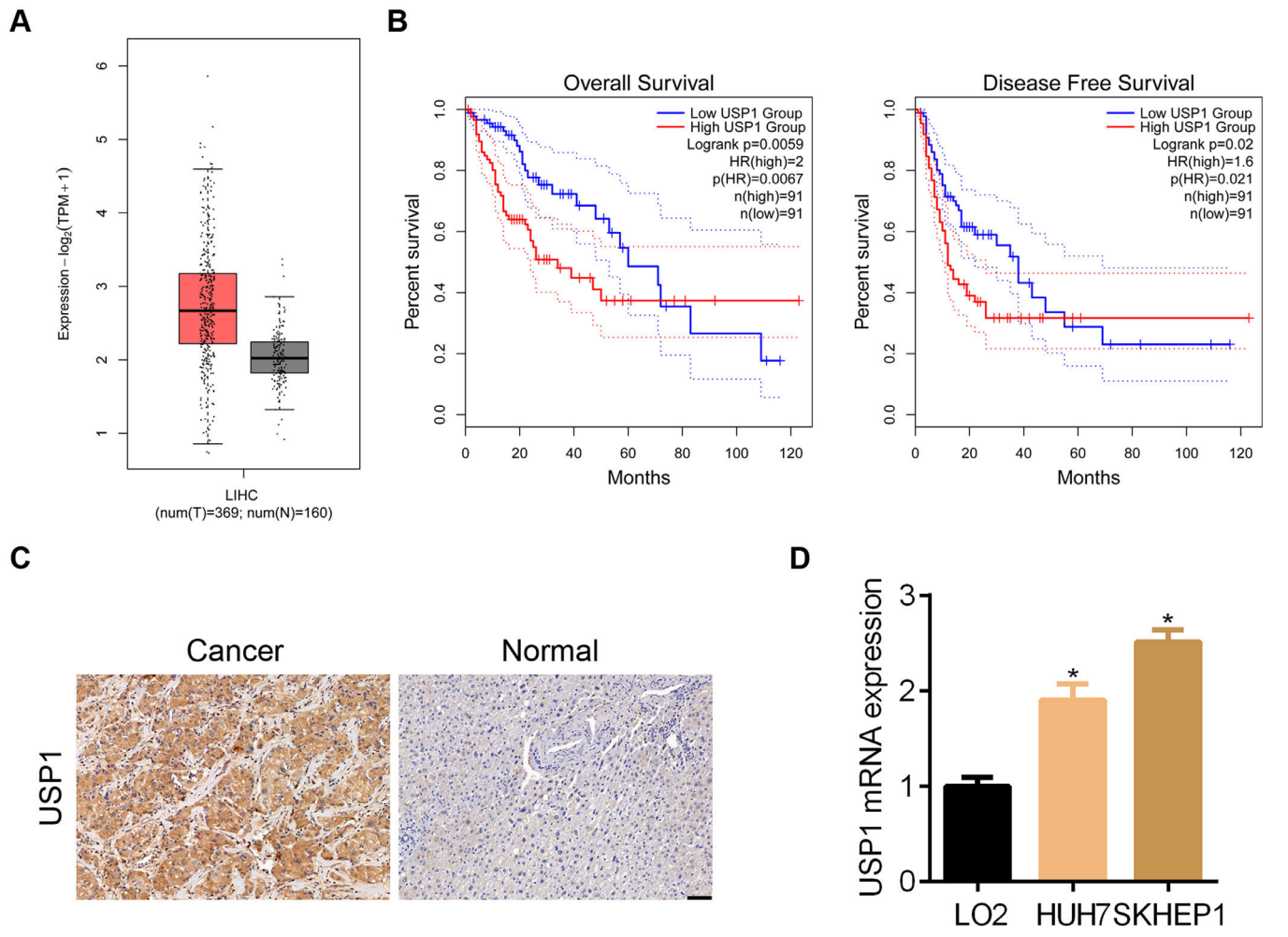

Fig. 1. The clinical relevance of USP1 in HCC patients.

(A) The transcript level of USP1 in HCC (n = 369) and normal (n = 160) tissues was analyzed based the RNAseq data from TCGA. P < 0.05. (B) HCC patients who were subdivided into USP1 high and USP1 low expression group. Overall and disease free survival was analyzed from TCGA. p value was presented in the Figure. (C) IHC staining of USP1 in human HCC tissue microarray, which contained HCC tissues and paired normal tissues. (D) mRNA expression of USP1 was checked in LO2, HUH7 and SKHEP1 cells. *p < 0.05.

HUH7 and SKHEP1 cells (Fig. 2B). After that, we examined the effect of USP1 overexpression by infecting the cells with lentivirus containing the coding sequence of USP1. Immunoblotting results showed that USP1 was overexpressed in HUH7 and SKHEP1 cells (Fig. 2C). In contrast to the effect of USP1 knockdown, USP1 overexpression could accelerate HCC cell growth (Fig. 2D). Moreover, USP1 overexpression in normal liver cells LO2 can also enhance the proliferative ability of the cells (Fig. 2E). We also performed colony formation assay to validate the oncogenic function of USP1. Down regulation of USP1 by siRNAs significantly suppressed the colony formation in HUH7 and SKHEP1 cells (Fig. 2F and 2G). These results suggest that USP1 reinforces HCC cell growth and

Table 1
The correlation between USP1 expression with HCC patients' characteristics based on TCGA database.

		USP1 expression		
	Characteristic	High	Low	p Value
Age	≤60	119	113	
	>60	65	73	0.435
AJCC stage	I/II	115	142	
	III/IV	55	35	0.007
Metastasis	M0	130	136	
	M1	1	3	0.343
T stage	T1/T2	128	147	
	T3/T4	57	36	0.014
Nodal status	N0	127	125	
	N1	3	1	0.329

Table 2
The correlation between USP1 expression with HCC patients' characteristics based on IHC staining.

		USP1 expression		
	Characteristic	High	Low	p Value
Age	<60	7	3	0.737
	≥60	5	3	
Total		12	6	
AJCC Stage	I/II	2	4	0.033
	III/IV	10	2	
Total		12	6	
Grade	G1/2	4	3	0.491
	G3/4	8	3	
Total		12	6	

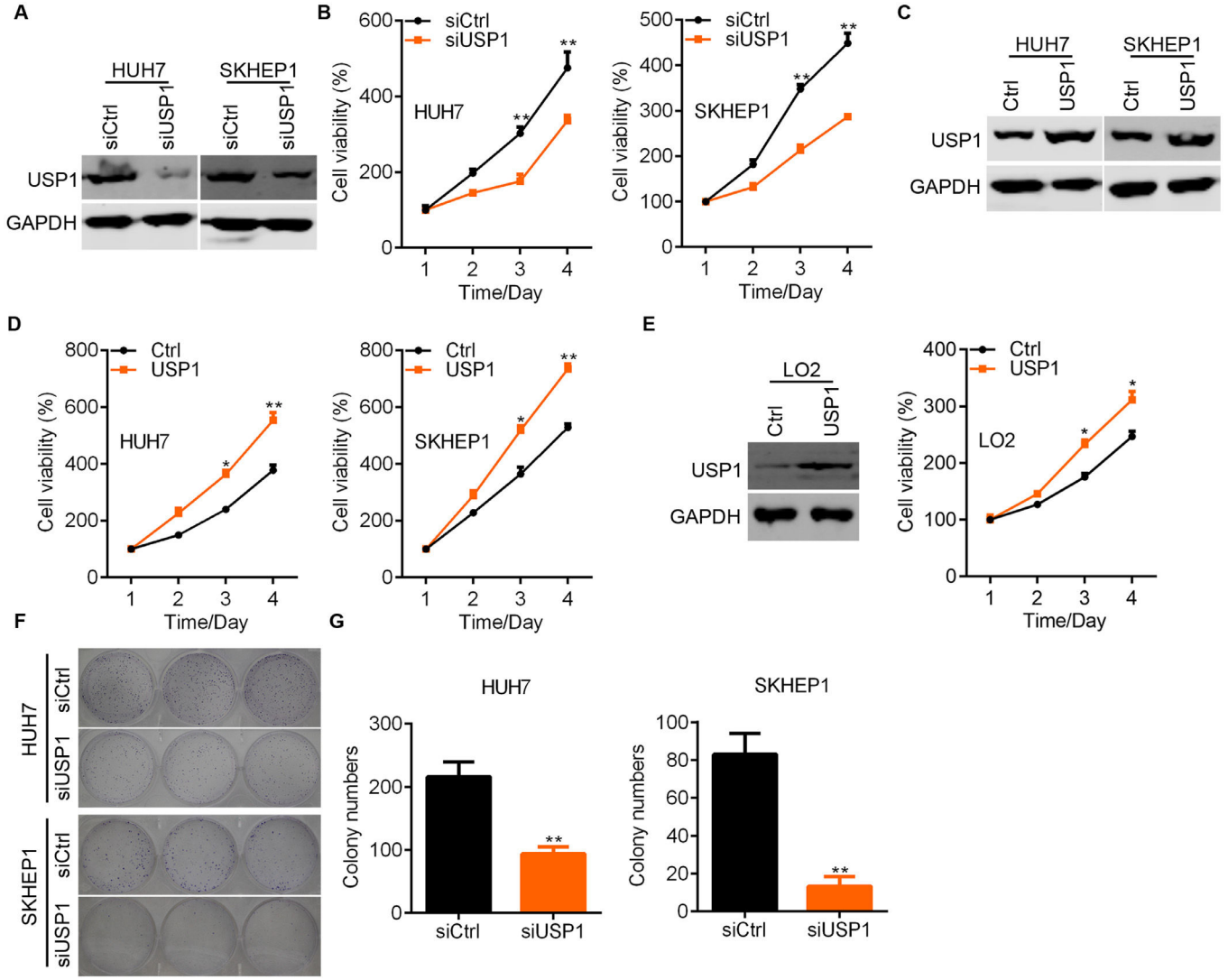

Fig. 2. USP1 acts as an oncogene in HCC.

(A) Immunoblotting results of USP1 in HUH7 and SKHEP1 cells transfected with siCtrl and siUSP1. GAPDH is the internal control. (B) Cell proliferation was measured in siCtrl and siUSP1 HUH7 and SKHEP1 cells by CCK8 assay. *p < 0.05. **p < 0.01. (C) Immunoblotting results of USP1 in HUH7 and SKHEP1 cells transfected with Ctrl and USP1 overexpression lentivirus. GAPDH is the internal control. (D) Cell proliferation was measured in Ctrl and USP1 overexpressed HUH7 and SKHEP1 cells by CCK8 assay. *p < 0.05. **p < 0.01. (E) Ctrl and USP1 overexpressed LO2 cells were subjected to immunoblotting analysis of USP1 and CKK8 analysis of cell proliferation. *p < 0.05. (F and G) Colony formation was examined in siCtrl and siUSP1 HUH7 and SKHEP1 cells. Quantitative results were shown in the Figure. **p < 0.01.

proliferation. Together with the clinical results, we propose that USP1 acts as an oncogene in HCC.

3.3. *c-kit is a potential target for USP1*

To examine the downstream target for USP1, we overexpressed USP1-Flag in HEK293 cells and performed immunoprecipitation with anti-IgG and anti-Flag. The lysates were then subjected to coomassie blue staining and mass spectrum. The results showed that USP1 might interact with c-kit, which is an oncogenic protein in various cancers (Fig. 3A and Table 3). We then performed immunoprecipitation experiments to check whether there was an interaction between USP1 and c-kit. Overexpression of USP1-Flag was constructed in HEK293 cells and we observed high overexpression efficiency (Fig. 3B). Positive immunoblotting results of c-kit were found in cell lysates of Input group and Co-IP of Flag group (Fig. 3B). Consistently, USP1 protein expression was also observed in cell lysates in which Co-IP was performed with Flag antibody (Fig. 3B). These results suggest that USP1 interacts with c-kit and promotes the protein stability of c-kit.

3.4. *USP1 stabilizes c-kit at post-transcription level*

USP1 is a deubiquitinating enzyme which can interact with and enhance the stability of downstream protein. We then wanted to address whether USP1 regulates c-kit in HCC cells. We found that USP1 down regulation or upregulation had no effect on mRNA expression of c-kit in HUH7 and SKHEP1 cells (Fig. 4A and 4B). By contrast, immunoblotting results showed that USP1 overexpression promoted, while USP1 knockdown suppressed the protein expression of c-kit (Fig. 4C and 4D). Furthermore, when MG132 treatment slightly reduced the protein abundance of c-kit in siCtrl HUH7 cells, it significantly promoted the protein degradation of c-kit in siUSP1 cells

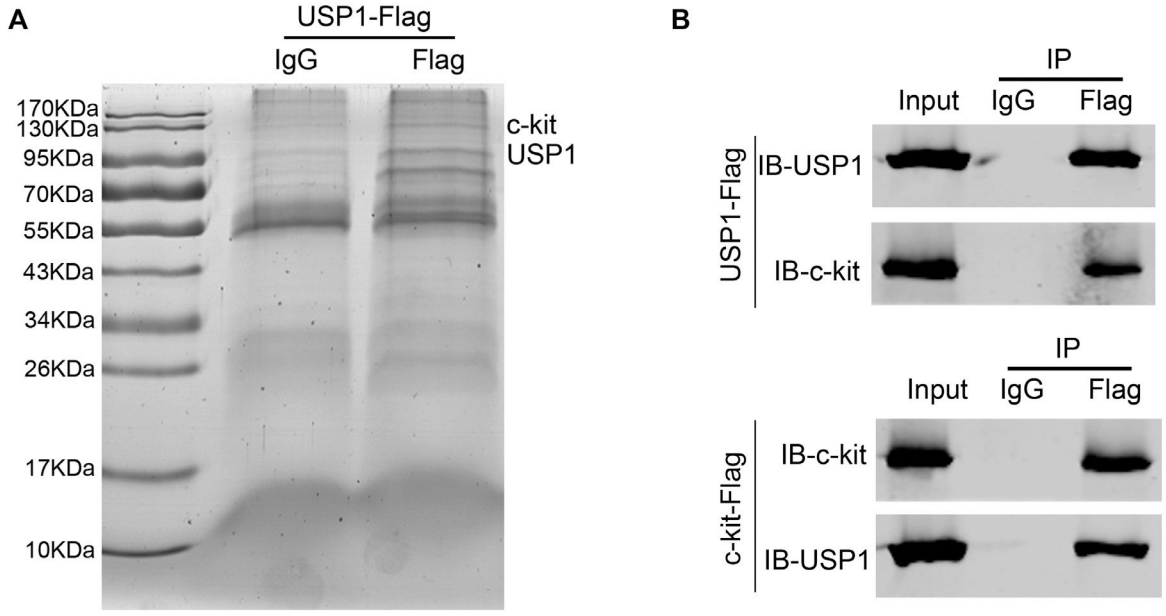

Fig. 3. c-kit is a potential target for USP1.

(A) USP1-Flag was overexpressed in HEK293 cells and subjected to immunoprecipitation with anti-IgG and anti-Flag. The immunoprecipites were visualized with coomassie blue. (B) USP1-flag was overexpressed in HEK293 cells. Immunoprecipitation was performed with IgG and Flag antibody. Protein lysates were subjected to immunoblotting with indicated antibodies. c-Kit-Flag was overexpressed in HEK293 cells. Immunoprecipitation was performed with IgG and Flag antibody. Protein lysates were subjected to immunoblotting with indicated antibodies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4E). Collectively, USP1 regulates the protein stability of c-kit in HCC cells.

3.5. Knockdown of c-kit reverses the oncogenic function of USP1 in HCC cells

To analyze whether USP1 promotes HCC cell growth through promoting the expression of c-kit, we silenced c-kit in USP1 overexpressed cells. Immunoblotting results showed that c-kit was efficiently silenced in USP1 overexpressed HUH7 and SKHEP1 cells (Fig. 5A). CCK8 results suggested that c-kit knockdown could reverse the promoting function of USP1 in HUH7 and SKHEP1 cells (Fig. 5B). Taken together, USP1 promotes HCC cell growth through upregulation of c-kit.

3.6. USP1 expression promotes the efficacy of lenvatinib in HCC cells

The activity or expression of c-kit predicts the efficacy of lenvatinib for the treatment of HCC patients. Since USP1 positively regulated c-kit expression in HCC cells, we lastly explored whether USP1 was correlated with lenvatinib efficacy. Ctrl and USP1 overexpressed HUH7 and SKHEP1 cells were incubated with different dosage of lenvatinib. In HUH7 cells, all concentrations of lenvatinib had larger inhibitory effect on cell viability of HUH7 cells with USP1 overexpression (Fig. 6A). Although there was no difference between Ctrl and USP1 overexpressed SKHEP1 cells when treated with 2.5 uM of lenvatinib, other concentrations, such as 5, 7.5, 10 and 20 uM, exhibited higher inhibitory effect on cell viability of SKHEP1 cells with USP1 overexpression (Fig. 6A). Lenvatinib also enhanced cell apoptosis in SKHEP1 cells with overexpression of USP1 (Fig. 6B). In summary, USP1 expression predicts lenvatinib efficacy in HCC cells.

Table 3
Top five proteins detected by mass spectrum.

Gene name	Molecular weight	Abundances (Normalized): IgG group	Abundances (Normalized): Flag group
USP1	95KDa	0	3008275.325
TGM3	77KDa	0	1932636.221
c-kit	130KDa	0	1658963.1
FXR1	68KDa	0	549257.25
CCT8	60KDa	0	393745.56

4. Discussion

Until now, surgical resection remains as the preferential treatment option for HCC patients. However, surgery treatment could only be effective for the patients with early diagnosed HCC. Despite targeted therapies, such as lenvatinib, have gained much progress and success on the treatment of HCC, a large population of HCC patients are resistant to this drug or relapse soon after the treatment. Therefore, identifying novel molecular biomarkers is urgent for the oncologists to formulate treatment criterion. In this study, we demonstrated that USP1 overexpression not only conferred the progression of HCC, but also enhanced the toxicity of lenvatinib on HCC cells by stabilizing c-Kit. Firstly, USP1 was highly expressed in HCC tissues. Overexpression of USP1 was correlated with shorter survival of HCC patients. Secondly, loss-of-function and gain-of-function experiments showed that USP1 contributed to HCC cell growth and proliferation. Thirdly, Co-IP and immunoblotting assays identified that USP1 interacted with and stabilized c-Kit. Lastly, USP1 stabilization of c-kit contributed to the increased toxicity of lenvatinib on HCC cells.

Ubiquitin-specific proteases (USPs) are one of the largest deubiquitinating enzyme families. Abnormal expression of USPs

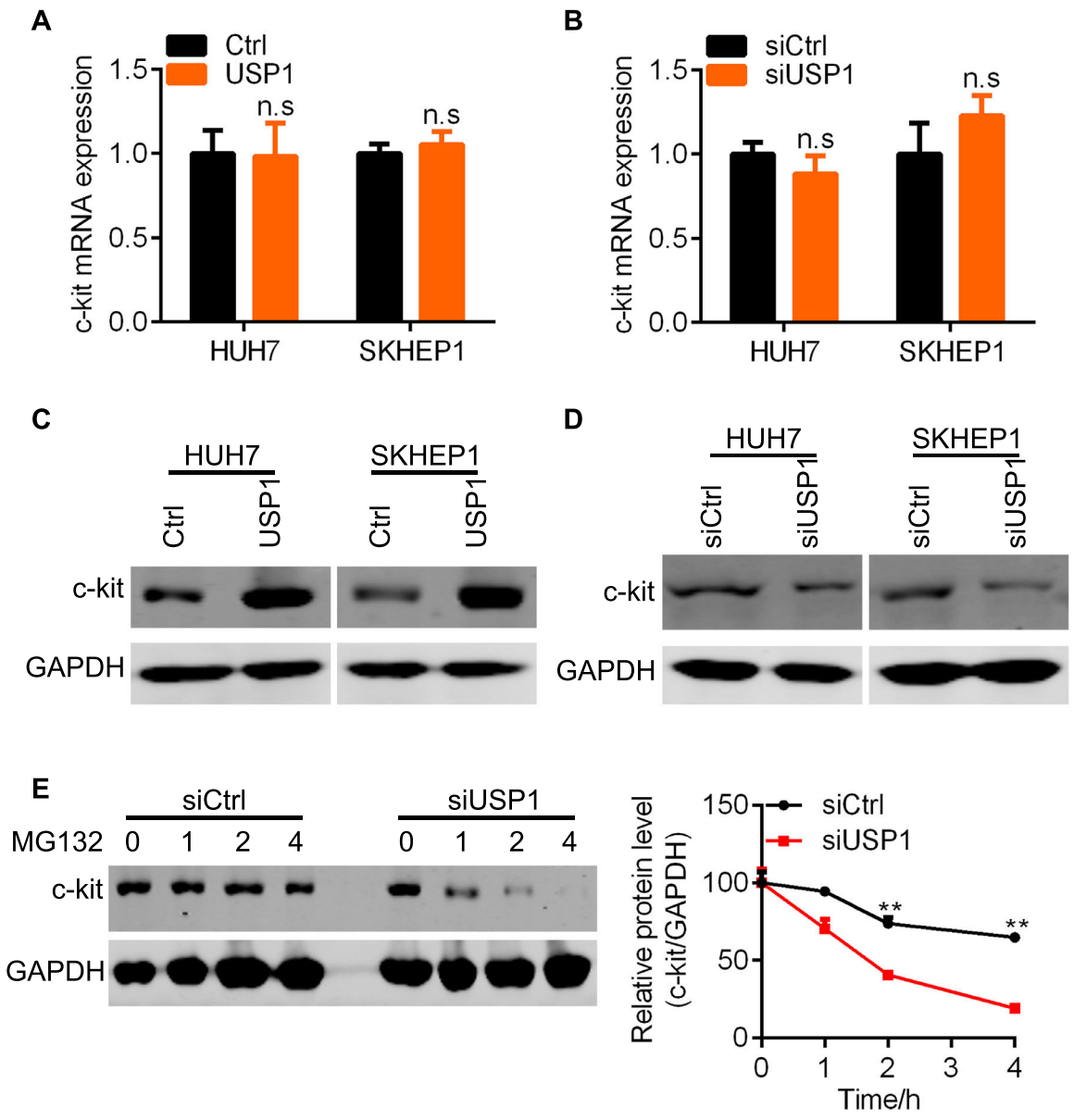

Fig. 4. USP1 stabilizes c-kit in HCC cells.

(A and B) mRNA expression of c-kit was checked in Ctrl and USP1 overexpressed (A), and in siCtrl and siUSP1 (B) HUH7 and SKHEP1 cells. n.s, no significance. (C) Immunoblotting results of c-kit in Ctrl and USP1 overexpressed cells. GAPDH is the internal control. (D) Immunoblotting results of c-kit in siCtrl and siUSP1 cells. GAPDH is the internal control. (E) siCtrl and siUSP1 HUH7 cells were treated with MG132 (10 uM) for 0, 1, 2, 4 h. The cells were subjected to immunoblotting analysis of c-kit. GAPDH is the internal control.

participates in cancer development through regulating different proteins. For example, USP2 deubiquitylation and stabilization of ErbB2 promotes the development of breast cancer. USP2 inhibitors can induce the sensitivity of ErbB2-positive breast cancer cells to HSP90 inhibitor treatment [14]. Suppression of USP2 also enhances chemotherapy effectiveness in triple negative breast cancer [15]. As a member of USPs, USP1 acts as an oncogene in various cancers. In breast cancer, USP1 promotes the protein stability of TAZ, a component of Hippo pathway, to induce breast cancer growth and metastasis [16]. A recent study has shown that USP1 potentiates ERα signaling activity during breast cancer progression [17]. In HCC, USP1 also functions as an oncogene. One comprehensive analysis identified that USP1 was overexpressed in HCC patients and correlated with the expression of various genes, including WDR48, cyclin D1, cyclin E1 and proliferating cell nuclear antigen (PCNA) [18]. Recently, USP1 was shown to contribute to the survival of circulating tumor cells by enhancing the protein stability of TBLR1 [19]. Nevertheless, the significance of USP1 in HCC cell growth and its correlation with lenvatinib treatment are limited understood. Here, we showed that USP1 overexpression was correlated with the prognosis of HCC patients. Expression of USP1 was critical for HCC cell proliferation. Overexpression of USP1 also promoted the growth of normal liver cells LO2. Furthermore, USP1 expression dictated the sensitivity of HCC cells to lenvatinib treatment. Our study revealed that USP1 functioned as an oncogene in HCC.

Lenvatinib is a multiple tyrosine kinase inhibitor which inhibits the activity of vascular endothelial growth factor (VEGF) receptors 1-3, fibroblast growth factor receptors (FGFR) 1-4, platelet-derived growth factor receptor-alpha (PDGFRα), RET and c-Kit [20]. Lenvatinib has been used for the treatment of wide

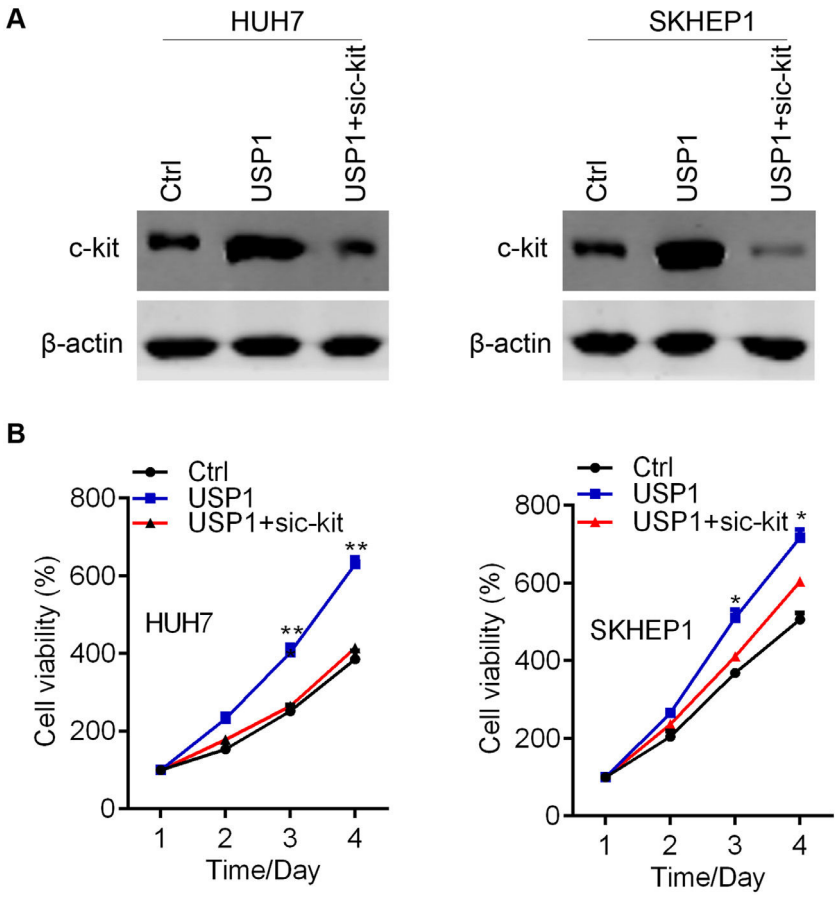

Fig. 5. USP1 upregulation of c-kit promotes HCC cell growth.
(A) Immunoblotting analysis of c-kit in Ctrl, USP1, and USP1+sic-kit HUH7 and SKHEP1 cells. (B) CCK8 analysis of cell proliferation in cells described in A. *$p < 0.05$. **$p < 0.01$.

range of cancers, including renal cell carcinoma [21], advanced thyroid cancer [22,23], and HCC [24]. c-Kit is a tyrosine-protein kinase that is amplified or overexpressed in HCC. Ectopic expression of c-Kit is a promising prognostic factor for HBV and HCV-associated HCC [25,26]. TGF-β/SMAD2 signaling pathway transcriptionally activates c-Kit to support HCC cell survival [27]. Even though numbers of HCC patients benefit from lenvatinib treatment, the biology basis and the precise role of c-Kit in lenvatinib efficacy should be determined. Because USP1 stabilized c-Kit at protein level, we predicted there was a correlation between USP1 and lenvatinib toxicity in HCC cells. As expected, USP1 overexpression enhanced the sensitivity of HCC cells to lenvatinib treatment. These result indicated that USP1 was a potential predictor for the efficacy of lenvatinib treatment.

Besides, there were some other potential interacting proteins of USP1, such as TGM3, FXR1 and CCT8. As reported, TGM3 is upregulated in HCC samples [28]. Overexpression of TGM3 promoted the EMT and malignant function of HCC [29]. CCT8 is also overexpressed in HCC specimens and contributes to the progression of HCC [30]. Unlike these two proteins, the significance of FXR1 in HCC is less reported. Based on these studies, we thought that USP1 might contribute to HCC through regulating these proteins, which needed further experiments to validate. This could explain why c-kit knockdown does not completely rescue the oncogenic role of USP1 in both HUH7 and SKHEP1 cells.

There were some limitations in this study. 1. We did not explore the correlation between USP1 and lenvatinib response in HCC patients. 2. We did not further investigate the role of other potential interacting proteins, such as TGM3, FXR1 and CCT8, in HCC development. 3. Although USP1 promoted the growth of LO2 cells, whether USP1 was critical for the initiation of HCC needed additional experiments. In the future, we will perform extra experiments to address these questions.

In conclusion, we provided the findings that USP1 was an oncogene in HCC by enhancing the expression of c-Kit. USP1 also dictated the sensitivity of HCC cells to lenvatinib treatment, which depended on its regulation of c-Kit expression. Our study highlights the important role of USP1 in HCC cell growth and proliferation. Targeting c-Kit with lenvatinib could be effective for the patients with highly expressed USP1. These novel findings provided potential clues for the researchers that USP1 was not only a drug target but also a predictor for lenvatinib response in HCC patients. In the future, it will be interesting for the researchers to deeply explore the precise function and molecular mechanisms of USP1 during HCC initiation and progression, as well as drug resistance, including targeted and ICIs therapy.

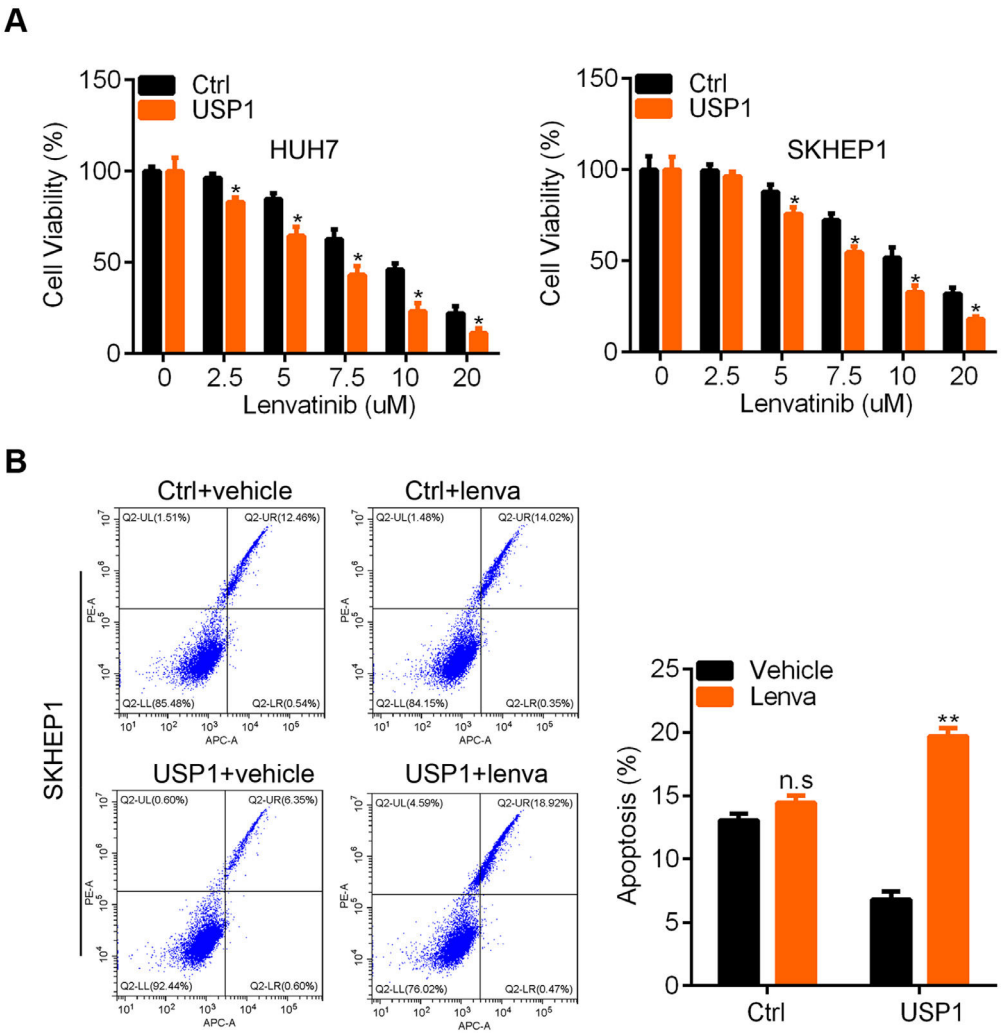

Fig. 6. USP1 enhances the efficacy of lenvatinib in HCC cells.
(A) Ctrl and USP1 overexpressed HUH7 and SKHEP1 cells were treated with 0, 2.5, 5, 7.5, 10 and 20 uM of lenvatinib for 48 h. Cell viability was assessed by CCK8 assay. *p < 0.05. (B) Ctrl and USP1 overexpressed SKHEP1 cells were treated with 5 uM of lenvatinib for 48 h. Apoptosis was measured by PI/Annexin V-APC staining.

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Author contributions

ZC, ZC and MS designed this study. ZC and YM performed most of the experiments. ZG and DS provided assistance. ZC, YM, ZC and MS analyzed the data and wrote the manuscript.

Conflicts of interest

These authors declared no competing interests.

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Not Applicable.

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