Ubiquitin-specific protease 11 serves as a marker of poor prognosis and promotes metastasis in hepatocellular carcinoma

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Abstract

Ubiquitin-specific protease 11 (USP11) is a deubiquitinating enzyme that exerts its biological functions by regulating multiple signaling pathways such as p53, NF- κ B, TGF- β , and Hippo. A large body of evidence supports a link between USP11 and tumorigenesis. However, the clinical significance and biological function of USP11 in hepatocellular carcinoma (HCC) remains unclear. Here, USP11 expression was assessed by immunohistochemistry in a pilot series of 71 HCC clinical samples, and the association between USP11 expression and clinicopathological features and overall survival time was analyzed. The cytoplasmic expression rate of USP11 was higher in non-cancerous tissue than that in cancer tissue (36.6 vs. 12.7%, $P = 0.001$), whereas the nuclear expression rate of USP11 was lower in non-cancerous tissue (5.6 vs. 69.0%, $P < 0.001$). USP11 expression level was higher in tumor than that in non-tumor tissue ($P < 0.001$). Chi-square analysis of variances suggested that USP11 expression was associated with vascular invasion ($P = 0.033$), differentiation ($P = 0.027$), tumor number ($P = 0.009$), and recurrence ($P = 0.036$). USP11 expression was also associated with shorter overall survival time ($P = 0.001$) by log-rank test. Unconditional logistic regression analysis with multiple covariates indicated that high USP11 expression was associated with a 2.96-fold increase in the risk of death compared with low USP11 levels ($P = 0.041$) and acted as an independent predictor of overall survival. HCC patients with simultaneously high USP11 and alpha-fetoprotein expression had an adjusted 5-fold higher risk of all-cause-related death ($P = 0.006$). Moreover, in vitro and in vivo experiments confirmed that USP11 could promote the migration and invasion of HCC cell. Overall, we suggest that USP11 promotes HCC cell metastasis, and we provide the first evidence of the prognostic significance of USP11 expression in HCC, which suggests that USP11 is a promising therapeutic target for the treatment of HCC.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and the second leading cause of cancer-related mortality [1]. Because of its aggressive nature and a high rate of recurrence and metastasis, the prognosis and survival of HCC patients remains disappointing [2]. Therefore, the identification of novel prognostic and predictive markers for clinical use is important.

Post-translational modification of proteins by covalent attachment of ubiquitin via the E1–E2–E3 ubiquitin ligation enzyme cascade is involved in the regulation of many essential cellular processes [3]. E3 ubiquitin ligases select substrates for ubiquitin conjugation, which is reversed by the action of deubiquitinating enzymes (DUB) [4–6]. At least 79 DUBs are encoded in the human genome, and they are responsible for editing and removing ubiquitin chains by cleaving the isopeptide bond. Ubiquitin-specific protease 11 (USP11) is a DUB that belongs to a USP family and harbors

two internal ubiquitin-like domains and an N-terminal domain present in USPs [6].

USP11 binds to several substrates, mediating their stabilization and deubiquitination. USP11 interacts with and stabilizes p53 by promoting its deubiquitination [7]. The deubiquitinase activity of USP11 is involved in the regulation of BRCA2 in response to DNA damage, leading to increased survival of cancer cells [8]. USP11 plays an important role in the downregulation of TNF α -mediated NF- κ B activation by modulating I κ B kinase α (IKK α) stability [9], and promotes transforming growth factor β -1 (TGF β -1) signaling through de-ubiquitination and stabilization of T β RII [10]. USP11 can also modulate TGF- β signaling by deubiquitinating the type I TGF- β receptor ALK5 [11]. USP11 was recently shown to regulate the Hippo pathway by modulating the VGLL4/YAP-TEADs regulatory loop [12]. These studies collectively indicate that USP11 exerts its biological function by regulating multiple signaling pathways such as p53, NF- κ B, TGF- β , and Hippo among others.

Previous studies reported that USP11 acts as a tumor suppressor through different downstream targets. USP11 represses lung adenocarcinoma cell migration and tumor formation by regulating Mgl-1 [13]. USP11 deubiquitinates and stabilizes the promyelocytic leukemia protein and inhibits multiple malignant characteristics of brain tumors [14]. However, a large body of evidence demonstrates that the activity of USP11 is tightly linked to tumorigenesis. USP11 interacts with and stabilizes X-linked inhibitor of apoptosis protein (XIAP), leading to the inhibition of cell anoikis and apoptosis, which in turn promotes breast cancer tumor initiation and progression [15]. In addition, counteracting USP11 inhibits the growth of pancreatic cancer cells [16]. Likewise, USP11 downregulation sensitizes human colon cancer cell lines to apoptosis and suppresses tumor growth in a xenograft model [17].

Recent pathological studies revealed a correlation between abnormal accumulation of USP11 and poor prognosis in breast cancer [15, 18]. Moreover, USP11 over-expression is frequently found in colorectal cancer [19, 20] and melanoma [21] and is correlated with poor survival [17]. However, the prognostic significance and expression pattern of USP11 in HCC have not been addressed to date. As USP11 is related to tumorigenesis through its impact on different signaling pathways, we hypothesized that its expression is correlated with clinical outcome and may be involved in HCC development and progression. The aim of this retrospective study was to investigate the expression pattern of USP11 in HCC by immunohistochemistry (IHC) and elucidate the potential relationship between USP11 expression and clinical outcomes and clinicopathological features in HCC. Additionally, we assessed whether USP11

influenced cell migration and invasion in vitro and in vivo. This information could advance our knowledge of the molecular basis of HCC development, which may lead to the identification of new and effective treatment alternatives.

Materials and methods

Clinical sample collection

Seventy-one HCC patients were enrolled between January 2013 and December 2015 at Zhongshan Hospital Xiamen University in China. All samples were collected from the Department of Hepatobiliary Surgery and stored in the Chronic Liver Disease Biological Sample Bank. Patients who received preoperative anticancer treatments, such as chemotherapy and radiotherapy, were excluded from our present analysis. The histological diagnosis of classical HCC was reviewed and confirmed by two pathologists, and the histopathological type and grade were determined using the criteria of the World Health Organization classification. Complete follow-up information of the 71 patients was obtained by phone conversations with the patients or their family members, and overall survival was defined as the time from the operation to HCC-specific death or last follow-up, whichever came first. Patients who died of other causes were censored for survival analysis. The study was approved by the Ethics Committee of the hospital, and informed consent was obtained from each recruited patient prior to surgery. All study procedures were carried out in accordance with the ethical standards of the Helsinki Declaration.

Hematoxylin–eosin (H&E) and IHC staining

Procedures were performed as previously described [22]. Briefly, 4 μ m sections were pretreated before H&E and IHC staining. After deparaffinization of slides in xylene and rehydration through a series of graded ethanol, an antigen retrieval step was performed. Endogenous peroxidase was quenched by incubation of the sections with 0.3% hydrogen peroxide for 15 min at room temperature. Nonspecific binding was blocked by incubation with nonimmune serum for 15 min at room temperature. The sections were then incubated with USP11 polyclonal antibody (1:5000, 22340-1-AP; Proteintech) at 4 °C overnight. Negative controls were prepared by non-immune rabbit IgG at the same dilution as the primary antibody. Biotinylated secondary antibody and diaminobenzidine were purchased from Maixin Biotechnology (Fuzhou, China). H&E staining was performed as previously described [23].

Fig. 1 Representative immunohistochemical (IHC) staining of USP11 in HCC tissue. USP11 was classified into four categories according to the intensity of IHC staining, including negative (**a**), weak (**b**), moderate (**c**), and strong (**d**) expression. Scale bars = 100 μ m

Immunostaining evaluation

Immunohistochemical evaluation of USP11 expression was performed as described previously [24]. The intensity and extent of USP11 immunostaining were evaluated for all samples under double-blinded conditions. The intensity was scored as 0 (no expression), 1 (weak expression), 2 (moderate expression) and 3 (strong expression) (Seen in Fig. 1). The percentage of positive staining was scored as 0 (no stain), 1 ($\leq 25\%$), 2 (26–50%), 3 (51–75%), or 4 ($\geq 76\%$). As shown in Table 1, the percentage and intensity of USP11 expression were multiplied to generate a final score ranging from 0 to 12. The score was used to dichotomize the samples. Assessments were performed independently by two researchers blinded to the clinical data. Discrepant scores between researchers (a difference in composite score

of ≥ 3 or a difference in high vs. low outcome score) triggered a third researcher to obtain a final expression score. Low USP11 expression was defined as a score < 5 ($n = 47$) and high USP11 expression was defined as a score ≥ 5 ($n = 24$).

Cell culture

Human liver cancer cell lines used in this study were MHCC-97h, PLC/PRF/5, HepG2, Huh7. The cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (Invitrogen), 100 IU/ml penicillin (Sigma, St Louis, MO, USA), and 100 mg/ml streptomycin (Sigma). Cells were grown on sterile cell culture dishes and passaged every 2–3 days using 0.25% trypsin (Invitrogen).

Small interfering RNA treatment

For target gene knockdown, control and shRNA sequences (shown in Table 2) against USP11 were cloned into the pLKO.1 interference vector. Then 293T cells were transfected with the above mentioned plasmids and the virus packaging plasmids using the Turbofect transfection reagent (Thermo Cat #R0531, R0532). The medium was changed 24 h after transfection and the medium containing the lentivirus was collected 48 h later. Cell lines were plated in six-well plates with full conditional media for 24 h prior to

Table 1 Immunohistochemistry scoring system for USP11 expression

Intensity	Score	Percentage of staining positive cells (%)	Score
Negative	0	No stain	0
Weak	1	≤ 25	1
Moderate	2	26–50	2
Strong	3	51–75	3
		≥ 76	4

For both scores, any stromal and inflammation cells or necrotic regions were excluded from analysis

Table 2 Primer sequences

	Primer name	F:5'-3'	R:5'-3'
shRNA	USP11-1	CCGGTGCCGTGACTA	AATTCAAAAACCGTG
		CAACAACCTCTACTC	ACTACAACAACCTCT
		GAGTAGGAGTTGTTG	ACTCGAGTAGGAGTT
		TAGTCACGGTTTTTG	GTTGTAGTCACGGCA
	USP11-2	CCGGTGCCGTGATGA	AATTCAAAAACCGTG
		TATCTTCGTCTACTCG	ATGATATCTTCGTCTA
		AGTAGACGAAGATAT	CTCGAGTAGACGAAG
		CATCACGGTTTTTG	ATATCATCACGGA
	USP11-3	CCGGTGCCCTCCCTT	AATTCAAAAACCTTC
		CTAGTCTTTATTCTCG	CCTTCTAGTCTTTATT
		AGAATAAAGACTAGA	CTCGAGAATAAAGAC
		AGGGAGGGTTTTTG	TAGAAGGGAGGGCA

infection. Infection was performed via virus-containing supernatant and Polybrene (10 mg/ml). Infected cells were cultured for selection with puromycin (1.2 µg/ml) after infecting twice. Stable transfectants were maintained in regular medium with puromycin (1.0 µg/ml) for further analysis.

Western Blot Analysis

Protein lysates were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Subsequently, the membrane was incubated with rabbit monoclonal antibody against human USP11 (1: 1000, 22340-1-AP, Proteintech), Alpha Tubulin (1:1000, 11224-1-AP, Proteintech) and Lamin B1 (1:1000, 12987-1-AP, Proteintech) at 4 °C overnight. After that, the blots were incubated with the secondary antibody labeled with horse radish peroxidase at room temperature for 2 h. Protein bands were visualized using enhanced chemiluminescence and quantified by densitometry using Image-J software. The relative protein levels were calculated by comparison to the amount of β-actin protein. Experiments were performed at least three times independently.

Migration and invasion assays

Cell migration and invasion assays were performed using Transwell chambers with or without Matrigel (8 µm, Corning), according to the manufacturer's instructions. In short, 2×10^5 cells were seeded in the upper chamber and 800 µl medium supplemented with 15% serum was added to the lower chamber (BD Biosciences, San Jose, CA, USA). After 48 h incubation, cells that had migrated to the lower side of the filter were fixed, stained with a dye solution, and counted. Experiments were performed in triplicate, and at least 10 fields were counted in each experiment.

CCK-8 assay

Approximately 1×10^4 cells per well with three repeats were plated into 96-well plates. Cell Counting Kit-8 (CCK-8) assays were performed using a commercial kit (#YB-K001, Guangzhou, China) according to the manufacturer's instructions. Optical density values were measured at a wave length of 450 nm using a microplate reader. All experiments were performed three times and the mean for each group was calculated.

RNA isolation and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was extracted from cells with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized using a cDNA Synthesis Kit (Tiangen, Beijing, China). Real-time PCR was performed on the Lightcycle Real-Time PCR System (Roche) using FastStart Universal SYBR Green Master (Roche). The sequences of the primer pairs are shown as followed: VEGF, forward: 5'-GGGCA-GAATCATCACGAAGT-3', reverse: 5'-GGCTTGAA-GATGTACTCGATCTC-3'; GAPDH, forward: 5'-CTTGGTATCGTGGAAGGACTC-3', reverse: 5'-AGTAGAGGCAGGGATGATGT-3'. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. Fold change was normalized to internal reference gene GAPDH.

Serum HBV and AFP detection

Serum HBV DNA levels were analyzed using quantitative real-time PCR (q-PCR) following the manufacturer's instructions of the HBV DNA PCR kit (SANSURE BIOTECH INC., Changsha, China). Serum AFP level was detected using an automatic electrochemical luminescence analyzer (Roche E170, Mannheim, Germany) according to

Fig. 2 The distribution of USP11 in HCC and non-cancerous liver tissue. USP11 was detectable in the cytoplasm of benign and malignant cells (**a** non-cancerous liver tissue; **b** cancerous tissue). **c** The cytoplasmic expression rate of the USP11 protein in non-cancerous tissue was higher than that in cancer tissue (36.6 vs. 12.7%, $P =$

0.001). USP11 was detected in the nuclei of tumor and non-tumor liver tissue (**d**, non-cancerous liver tissue; **e**, cancerous tissue). **f** Compared to normal liver tissue, the nuclear expression rate of USP11 was significantly higher in HCC samples (69.0% vs. 5.6%, $P < 0.001$). Scale bars = 100 μm

the manufacturer's instructions of a commercially available chemiluminescence immunoassay kit (Roche Diagnostics GmbH, Germany). The electrochemiluminescence automatic immunoassay analyzer is based on paramagnetic particles as a solid phase, a biotin-streptavidin-detection system, and two-dimensional bar code technology.

Animal studies

In order to verify the results found at the cellular level, animal studies were performed with 2×10^5 cells injected into the subcutaneous of nude mouse, 8 cases for 97H-shCtrl group and 97H-ShUSP11 group, respectively. The mice were sacrificed 6 weeks later and the lung and liver tissue were collected for metastatic foci examination via standard pathological study. In short, the tissue was fixed with 10% neutral formalin and tissue blocks were cut into 4 μm sections and further for H&E stain. Animal work was performed on an approved Institutional Animal Care and Use Protocol of Xiamen University.

Statistical analysis

Statistical analyses were performed using SPSS 21.0 (IBM, Chicago, IL, USA) and GraphPad Prism 5.0 (La Jolla, CA,

USA) software. The Pearson chi-square test was used to analyze the relationship between USP11 expression and various clinicopathological parameters. Differences in USP11 immunostaining score between cancer and para-cancer tissue was evaluated with the Wilcoxon matched pairs test. Kaplan–Meier survival function was calculated and compared with the log-rank test. The Cox proportional hazard regression model was used for multivariate analyses to explore the effects of the clinicopathological variables and USP11 expression on patient survival. All statistical tests were two-tailed. A P value < 0.05 was considered significant.

Results

Patient characteristics

The clinical characteristics of the 71 HCC patients are as follows: the mean age of the patients was 53 years (range 20–90 years). Of them, 80.3% ($n = 57$) were men, 9.9% ($n = 7$) had poor differentiation according to the pathologist's diagnosis, 64.8% ($n = 46$) had tumors diameter larger than 5 cm, and 26.8% ($n = 19$) with multiple tumors. In addition, 62.0% ($n = 44$) of them had vascular invasion, 57.7% ($n =$

Fig. 3 Representative expression pattern of USP11. The representative expression pattern of USP11 in one paired tumor and non-tumor liver tissue (**a** H&E; **b** IHC). Scale bars = 200 μ m. USP11 was weakly expressed in non-cancerous liver tissue (**c** H&E; **d** IHC), while it was

strongly expressed in cancer tissue (**e** H&E; **f** IHC). **g** Statistical analysis of IHC staining scores in HCC and paracancer tissue by Wilcoxon's test ($P < 0.001$). Scale bars = 100 μ m. H&E hematoxylin and eosin, IHC immunohistochemistry

41) and 32.4% ($n = 23$) had serum alpha-fetoprotein (AFP) and hepatitis B virus (HBV) levels of <200 ng/ml and < 1000 Iu/ml, respectively; 43.7% ($n = 31$) had liver cirrhosis and 49.3% ($n = 35$) developed recurrence.

USP11 subcellular localization

To determine the subcellular localization of USP11 in HCC, IHC was performed in 71 cancerous and corresponding para-cancerous paraffin-embedded tissue archived from postoperative surgical samples. USP11 was detectable in the cytoplasm of benign and malignant cells (Fig. 2a, b). The cytoplasmic expression rate of the USP11 protein was higher in non-tumor tissue than that in tumor tissue [36.6% (26/71) vs. 12.7% (9/71), $P = 0.001$, Fig. 2c]. USP11 was also detected in the nuclei of tumor and non-tumor liver tissue (Fig. 2d, e). USP11 was expressed in the nucleus in 5.6% (4/71) of non-cancerous tissue, whereas nuclear expression was significantly higher in HCC samples at 69.0% (49/71) ($P < 0.001$, Fig. 2f). Moreover, we used a nucleoplasm separation method to detect the subcellular

location of USP11 in HCC and LO₂ cells, and the results showed that USP11 was mainly located in HCC cell nucleus, while in LO₂ cells, USP11 was mainly located in the cytoplasm (Supplementary Figure S1).

Expression of USP11

To understand the possible role of USP11 in the development and progression of HCC, we examined the expression pattern of this protein in paired clinical tissue samples (Figure 3a and b). As shown in Fig. 3c and d, USP11 was undetectable or expressed very weakly in non-cancerous liver tissue. Among the 71 paracancer tissue samples, the IHC score of USP11 was <3, whereas 40.8% of tumor tissue showed relatively higher USP11 expression (29/71 cases, Fig. 3e, f). Overall, the IHC score of USP11 in cancer tissue was significantly higher than that in paracancer tissue ($P < 0.001$, Fig. 3g). Additionally, we examined the expression patterns of the USP11 protein in HCC clinical specimens by Western blotting. Consistent with the immunohistochemistry results, Western blot analysis

Table 3 Association between USP11 expression by IHC and clinicopathological features of HCC

Clinical-pathology variable	USP11 high	N	X ²	P value
Age (year)				
<53	12(34.3%)	35	0.007	0.932
≥53	12(33.3%)	36		
Gender				
Male	18(31.6%)	57	0.639	0.424
Female	6(42.9%)	14		
Tumor size				
<5 cm	8(32.0%)	25	0.056	0.813
≥5 cm	16(34.8%)	46		
Vascular invasion				
Yes	19(43.2%)	44	4.548	0.033 ^a
No	5(18.5%)	27		
Differentiation				
Poor	5(71.4%)	7	4.913	0.027 ^a
Moderate-well	19(29.7%)	64		
Liver cirrhosis status				
No	10(25.0%)	40	3.173	0.075
Yes	14(45.2%)	31		
Serum HBV level (Iu/mL)				
<1000	7(30.4%)	23	0.172	0.678
≥1000	17(35.4%)	48		
Serum AFP level (ng/mL)				
<200	13(31.7%)	41	0.190	0.663
≥200	11(36.7%)	30		
Tumor number				
Multiple	11(57.9%)	19	6.729	0.009 ^a
Single	13(25.0%)	52		
Recurrence				
Yes	16(45.7%)	35	4.377	0.036 ^a
No	8(22.2%)	36		

IHC immunohistochemistry, HCC hepatocellular carcinoma, HBV hepatitis B virus, AFP a-Fetoprotein

^aStatistically significant, $P < 0.05$

showed that USP11 protein expression was significantly up-regulated in HCC tissue compared with adjacent normal tissue ($P < 0.001$, Supplementary Figure S2). Additionally, we examined USP11 expression pattern in some commonly used HCC cell lines including Huh7, PLC/PRF/5, HepG2, and MHCC-97H, and the results showed that USP11 expression levels were consistently increased in HCC cell lines (Supplementary Figure S2).

Association of USP11 expression with clinicopathological features of HCC patients

The results of USP11 IHC staining of HCC tissue samples according to the clinicopathologic characteristics of the

patients are summarized in Table 3. The rate of high USP11 expression in patients with vascular invasion (19/44, 43.2%) was higher than that in patients without invasion (5/27, 18.5%) ($P = 0.033$). The rate of high USP11 expression was higher in patients with poorly differentiated tumors (5/7, 71.4%) than in patients with moderate to well differentiated tumors (19/64, 29.7%) ($P = 0.027$). The rate of high USP11 expression was higher in patients with multiple tumors (11/19, 57.9%) than in those with a single tumor (13/52, 25.0%) ($P = 0.009$). Higher expression of USP11 was associated with recurrence ($P = 0.036$). No significant association was found between USP11 protein expression and age, gender, tumor size, liver cirrhosis status, and serum HBV and AFP levels ($P > 0.05$, Table 3). These results indicate that USP11 expression may be correlated with the development and progression of HCC.

Expression of USP11 predicts unfavorable prognosis of HCC patients

To investigate the prognostic value of USP11 expression, the association of USP11 protein expression with clinical outcomes of HCC patients was analyzed. Statistical analysis showed that the 1-year survival rates were 87.5% for USP11-low patients and 63.6% for USP11-high patients ($P = 0.027$, Fig. 4a). Kaplan–Meier analysis confirmed that patients with high USP11 expression had a shorter overall postoperative survival time than those with low USP11 expression ($P = 0.001$, Fig. 4b). The Cox proportional hazards regression model was used to determine whether abnormal USP11 expression was associated with patient prognosis. The results showed that high expression of USP11 was an independent risk factor associated with the prognosis of patients with HCC [hazard ratio (HR) = 2.962, with 95% confidence interval (CI) = 1.043–8.409; $P = 0.041$, Table 4]. Thus, USP11 predicted the outcomes of HCC patients and can be used as a biomarker for patient prognosis.

Effect of the combination of USP11 and AFP expression on outcomes

The effects of the combination of USP11 and serum AFP expression on outcomes are shown in Fig. 5. USP11-high/AFP-high vs. USP11-low/AFP-low ($P = 0.002$) and USP11-high/AFP-high vs. USP11-low/AFP-high ($P = 0.005$) patients showed significant differences in overall survival. The worst outcome was seen in USP11-high/AFP-high patients. In addition, univariable models demonstrated that relative to patients with USP11-low and AFP-low expressing cancers, patients with liver cancers showing high USP11 and AFP expression had a five-fold increased risk of

Fig. 4 Expression of USP11 and survival analysis. **a** Statistical analysis showed that the 1-year survival rate was 87.5% for patients with USP11-low and 63.6% for patients with USP11-high ($P = 0.027$). **b** Kaplan–Meier estimation performed on all HCC patients indicated that

patients with USP11-high expression ($n = 24$) have shorter overall survival time than patients with USP11-low expression ($n = 47$) ($P = 0.001$)

Table 4 Cox regression analysis of prognostic factors for HCC patients

Variables	P value	HR	95% CI	
			Lower	Upper
Age	0.727	0.823	0.277	2.449
Gender	0.644	1.387	0.347	5.538
Tumor size	0.131	0.383	0.110	1.332
Differentiation	0.570	0.658	0.156	2.781
Multiple tumor	0.715	1.246	0.383	4.049
Vascular invasion	0.021 ^a	.086	.011	.693
Liver cirrhosis status	0.409	.654	.238	1.794
Serum HBV level	0.518	.662	.189	2.314
Serum AFP level	0.607	1.295	.483	3.471
USP11 expression	0.041 ^a	2.962	1.043	8.409

HCC hepatocellular carcinoma, CI confidence interval, HBV hepatitis B virus, AFP, a-Fetoprotein

^aStatistically significant, $P < 0.05$

all-cause-related death (HR = 5.085, 95% CI = 1.600–16.164, $P = 0.006$, Table 5).

USP11 promotes HCC cell invasion and migration ability both in vitro and in vivo

To explore the biological function of USP11 in HCC cell invasion and migration potency, we used shRNAs to knockdown endogenous USP11 expression in the MHCC-97h and PLC/PRF/5 cell lines. When the above two cells were transfected with USP11 shRNAs, the expression of USP11 was dramatically reduced at the protein level (Fig. 6a and Supplementary Figure S3). Migration and invasion experiments showed that downregulation of

Fig. 5 Overall survival according to the combined effect of USP11 and AFP expression. USP11-high/AFP-high vs. USP11-low/AFP-low ($P = 0.002$) and USP11-high/AFP-high vs. USP11-low/AFP-high ($P = 0.005$) patients showed significant differences in overall survival. The worst outcome was seen in USP11-high/AFP-high patients

Table 5 Associations between USP11 and AFP status and time to death

	n	HR	P-value	95% CI	
				Lower	Upper
USP11-high/AFP-high	11	5.085	0.006 ^a	1.600	16.164
USP11-high/AFP-low	13	3.082	0.065	0.934	10.175
USP11-low/AFP-high	19	0.834	0.805	0.199	3.502
USP11-low/AFP-low	28	Reference			

CI confidence interval, HR hazard ratio, AFP a-Fetoprotein

^aStatistically significant, $P < 0.05$

Fig. 6 Silencing USP11 inhibits the metastasis of HCC cells. **a** The protein level of USP11 was significantly decreased following transfection with ShRNA lentiviruses compared with empty vector. **b** Representative images and quantification of migrated and invaded cells analyzed using the Matrigel invasion assay. A significant reduction in the invasive ability was observed in response to USP11 knockdown. The cell number was counted in five random view fields.

Scale bars = 100 μ m **c** Representative images of pulmonary and hepatic metastases in nude mice subcutaneously injected with control and USP11 knockdown MHCC-97h cells obtained by performing HE staining and results showed that the mice injected with control cells displayed more pulmonary and hepatic micro-metastatic nodules than mice injected with MHCC-97h-shUSP11. Scale bars = 100 μ m (top) and scale bars = 200 μ m (below) (* P < 0.05, *** P < 0.001)

USP11 significantly inhibited HCC cell migration and invasion ability (Fig. 6b). Based on this observation, we next determined the effects of USP11 on HCC metastasis *in vivo*. We used a xenograft mouse model that was generated by subcutaneously injecting control and MHCC-97H-shUSP11 cells into nude mice. Six weeks after implantation, tested mice were sacrificed and the metastatic nodules were counted. As seen in Fig. 6c, the mice injected with 97H-shUSP11 cells displayed less pulmonary and hepatic micro-metastases nodules which were confirmed via histological study and H&E stained compared with the mice injected with sh-control cells. Together, the results of *in vitro* and *in vivo* assays indicate that USP11 promoted the migration and invasion of HCC cells.

Discussion

HCC is the second most common malignancy and an important health problem worldwide. Despite advances in treatment modalities, no effective cure for this disease has been identified to date. The prognosis of HCC patients is often poor because of the lack of effective predictive markers. Therefore, the identification of prognostic markers among pretreatment variables, and in HCC biopsy specimens in particular, is critical.

The results of the present study demonstrated that high levels of USP11 contributed to poor clinical outcomes in HCC patients. USP11 expression was associated with vascular invasion, poor differentiation, tumor number, and recurrence. Multivariate logistic regression analysis showed, for the first time, that high USP11 expression was an independent risk factor for HCC patient outcome, which is consistent with a previous study in breast cancer [18]. However, several limitations must be considered when interpreting the results of our study. Our study was a retrospective analysis with a modest sample size and limited follow-up data. Because of the small sample size, we were unable to evaluate the prognostic significance of USP11 in a separate cohort of HBV-negative tumors. The small sample size also prevented the accurate evaluation of the relationship between the sub-cellular distribution of USP11 expression in HCC and the clinic-pathological characteristics. Additionally, these results were obtained in a local cohort and should be confirmed in other populations of HCC patients in further work. Nevertheless, it is an important pilot study in that it identifies the relationship between USP11 expression and the prognosis of HCC patients. Currently, there is an increased use of two or more biomarkers in the assessment of patient prognosis and response to treatment. To further determine the correlation between combined expression of USP11 and AFP with clinical outcome in HCC, we analyzed clinical information

of the 71 HCC cases included and demonstrated that HCC patients with simultaneously high USP11 and AFP expression have an increased risk of post-operative death in the form of shorter overall survival time.

A growing body of evidence suggests that mRNA and protein expression measurements obtained by quantitative polymerase chain reaction and Western blotting are contaminated by stromal and inflammatory cells within parenchyma cells. IHC analyses of USP11 expression would be more informative and was performed in the present study. Our results demonstrate that USP11 was expressed at high levels in the nucleus in HCC tissue. Consistent with our findings, Lin et al. reported that USP11 is mainly localized in the nucleus and reduces the ubiquitination level of E7 [25]. Ideguchi et al. concluded that USP11 is a ubiquitous protein that interacts with RanBPM and localizes to the nucleus [26]. By contrast, some studies showed that USP11 was highly expressed in the cytoplasm of breast cancer cells [18]. Hence, organ specificity and cell heterogeneity should be taken into consideration in the evaluation of USP11 location and status in tumor tissue. These apparent discrepancies between USP11 distribution in different tumors and clinical outcomes suggest that USP11 has different functions in each organ system, and thus may have a specific role in each organ's tumor biology.

Previous researchers have shown that USP11 forms a specific complex with p53 and stabilizes p53 by deubiquitinating it. Sun et al. found that USP11 inhibits I κ B α ubiquitination and the nuclear translocation of p65, which further suppress NF- κ B signal pathway activation. Moreover, Jacko et al. showed that USP11 promotes TGF β -1 signaling through de-ubiquitination and stabilization of T β RII. Additionally, USP11 was functions as a new regulatory layer of the Hippo pathway by deubiquitinating and stabilizing VGLL4 protein. Therefore, it is possible that a similar relationship exists between USP11 and p53, NF- κ B, TGF- β , and/ or Hippo in HCC. It would be important to explore this possibility and the potential mechanisms underlying the role of USP11 in regulating HCC cell migration ability.

A previous report showed that USP11 could deubiquitinate, stabilize, and combine with RanBPM, which has been well characterized as a molecule required for nuclear protein import and export [26]. Another study indicated that USP1 forms a complex with UAF1 in the cytoplasm, and this complex translocates to the nucleus through two nuclear localization signals (NLS) on USP1 [27]. USP11, as a homolog of USP1 and a member of the deubiquitinating enzyme family, also contains a potential NLS at amino acids 445–452 via bioinformatic analysis [28]. Herein, we speculated that NLS present within the domain of USP11 mediates its translocation into the cell nucleus and further acts as a tumor promoter, while the NLS sequence in non-

cancerous is either abrupt or inactivated. Yamaguchi et al. showed that knocking down USP11 attenuates the expression of IKK α at the transcriptional, but not the post-translational level, in a ubiquitin-independent manner [29]. We further speculated that USP11 translocates into the nucleus and acts as a transcription factor involved in oncogene activation, suggesting that it plays a critical role in HCC development and progression. However, further studies are needed to clarify this issue.

In the present study, we demonstrated that USP11 positively regulated HCC cell invasion and migration ability in vitro and in vivo. As we all know, angiogenesis is a key factor in tumor growth and metastasis. Hence, in our present study, we employed real-time PCR methods to analyze the VEGF mRNA level in the cell lines with altered USP11 expression. Our data showed that the VEGF mRNA level was significant down-regulated after USP11 protein was silenced (Seen in Supplementary Figure S4). These results suggesting that USP11 promoted HCC tumor invasion maybe through inducing angiogenesis. What's more, in order to confirm that the differences were not due to lack of viability after USP11 silencing, we performed a CCK-8 experiment to detect cell viability. As shown in supplementary Figure S5, there was no significant difference between the four groups after USP11 silencing for 48 h. After 48 h of incubation, migration and invasion experiment were performed, and the results demonstrated that USP11 positively regulates HCC cell invasion and migration ability. Additionally, USP11 promotes HCC cell proliferation in vitro (Supplementary Figure S5).

In recent years, there has been growing interest in finding new compounds to affect novel therapeutic targets and diseases [30, 31]. Mitoxantrone, a specific inhibitor of USP11, has been used for the last decade in the treatment of acute myeloid leukemia, hormone refractory prostate cancer, and multiple sclerosis [32]. To the best of our knowledge, this is the first report to document that high expression of USP11 correlates with worse prognosis in HCC. Our study provides evidence supporting USP11 as a promising “druggable” target and biomarker and the use of mitoxantrone as a bioavailable lead compound for the treatment of this lethal disease.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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