



# Overexpression of Ubiquitin-Specific Protease 15 (USP15) Promotes Tumor Growth and Inhibits Apoptosis and Correlated With Poor Disease-Free Survival in Hepatocellular Carcinoma

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## Abstract

USP15 is a member of ubiquitin-specific proteases (USPs, the largest subfamily of deubiquitinases) and functions as a stabilize factor of target proteins in reversible ubiquitination progression. Dysregulated expression of USP15 has been observed in various cancers. However the expression profile and regulatory mechanism of USP15 in hepatocellular carcinoma (HCC) remains largely elusive. To exam the USP15 expression changes in the progression of HCC, we performed IHC analysis to test USP15 expression in a series of cancer-prone diseases including 2 normal liver tissues, 6 liver cirrhosis, 16 primary liver lesions and 15 metastases of hepatocellular carcinoma. The expression of USP15 was upregulated in various liver diseases in compared with normal tissue significantly ( $p < 0.05$ ). Although no significant different of USP15 expression were discovered between cirrhotic tissue and primary tissue, its expression in HCC metastatic tissue was upregulated. Subsequently, we test the USP15 expression profile in a cohort of 66 HCC patients. USP15 expression was positively correlated with the recurrence of HCC significantly ( $p = 0.004$ ). HCC patients with high USP15 expression had shorter disease free survival time in compare with those with low USP15 expression (56.9% VS 26.7%,  $P = 0.012$ ). Subsequently, Cox multivariate analyses of clinical factors associated with disease free survival were performed and USP15 expression ( $p = 0.008$ ) together with tumor size ( $p = 0.034$ ) were proved to be independent predict factors in HCC. Then, we silenced USP15 expression in HCC cells and the results showed that downregulated USP15 expression resulting proliferation inhibition and apoptosis induction. In conclusion, our results suppose USP15 to be a potential target in HCC.

## Keywords

USP15 expression, hepatocellular carcinoma, metastasis, survival, potential target

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## Introduction

Liver cancer is the sixth commonly diagnosed cancer and fourth mortality worldwide behind lung cancer, colorectal cancer and stomach cancer.<sup>1</sup> Hepatocellular carcinoma (HCC) is the most common diagnosed liver cancers. Although the survival time of patients with HCC could be partially prolonged by surgery or chemotherapy, the mortality of HCC was still high due to recurrence or metastasis. Therefore, it is urgent to discover new molecular markers for HCC diagnosis and therapy to improve the outcome of HCC patients.

Ubiquitination is a reversible post-translational modification that target proteins for degradation. Deubiquitinases

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(DUBs) are responsible for stabilization of proteins. Ubiquitination was participated in many cellular life activities such as cell cycle regulation, DNA repair and signal transduction.<sup>2</sup> Dysregulation of ubiquitination was discovered in many diseases including cancer. Ubiquitination specific protease (USP) is the largest subfamily of deubiquitylase, which contains about 56 members. USPs share the same structures as 2 short and conserved fragments, namely Cys box and His box.<sup>3</sup>

USP15 belongs to USP, the largest subfamily of DUBs. Previous studies revealed that USP15 played dual roles of oncogenes and/or tumor suppressors in various tumors. Overexpression of USP15 has been reported in glioblastoma, breast cancer, ovarian cancer and myeloma, while downexpression has been identified in pancreatic cancer. Potential substrates for USP15 have been identified including many cancer related proteins, such as human papilloma virus E6 oncoprotein, adenomatous polyposis coli tumor suppressor, nuclear factor of light poly peptide gene enhancer in B cells inhibitor, TGF- $\beta$  and its receptor-regulated SMAD effectors, p53, Nrf-1 and Nrf-2. These various substrates for USP15 suggest that it played important role in cancer progression.<sup>4-6</sup>

Recent years, USP15 and USPs have attracted attention as potential therapeutic targets for various cancers. However, the underlying regulatory roles and mechanisms of USP15 in hepatocellular carcinoma, remain largely elusive.<sup>7</sup> In this study, we tried to reveal the molecular and clinical parameters association of USP15 expression and potential regulation mechanism in HCC.

## Materials and Methods

### Clinical Samples

A tissue microarray (Shanghai Outdo Biotech Co., Ltd.) that contained 2 normal live tissues, 6 cases of liver cirrhosis, 17 primary liver lesions and 15 metastases of hepatocellular carcinoma were used to investigate the expression of USP15 in different course of hepatocellular carcinoma.

66 tumor tissues from HCC patients with follow-up time of 4 years, were used to study the relationship between USP15 expression and hepatocellular carcinoma (Shanghai Outdo Biotech Co., Ltd.). The age of these patients ranged from 36 to 79, and the median age was 56.5. The detailed clinical information could be found in Table 1.

### Immunohistochemistry

Two-step immunohistochemistry was used in our study. Firstly, tissue sections were treated to retrieval antigen using EDTA buffer; and then tissues were incubated with primary antibody anti-USP15 (1:800, Proteintech) at 4°C overnight. Next sections were incubated with secondary antibody (HRP-labeled anti-mouse antibody, DAKO), then visualized using diaminobenzidine (DAB) and hematoxylin re-dyeing after washed with PBS. The immunohistochemical results were evaluated by pathologists, and scored as follows: negative for 0,

**Table 1.** Clinical Parameters of HCC Patients.

Clinical parameters	No. patients
Gender	
Male	57
Female	9
Age	
$\leq 60$	43
$> 60$	23
Tumor size	
$\leq 5\text{cm}$	44
$> 5\text{cm}$	22
Pathological grade	
II	46
III	20
T stage	
T1	45
T2	14
T3	7
N stage	
N0	65
N1	1
M stage	
M0	65
M1	1
cTNM stage	
1	43
2	14
3	7
4	2

“+” for 1, “++” for 2 and “+++” for 3. The positive staining rate was defined according to the proportion of positive stained cancer cells: “Negative” is 0, “1%-20%” for 1, “21%-40%” for 2, “41-60%” for 3, “61-80%” for 4, “81-100%” for 5. Take the product of “dyeing intensity” score and “dyeing positive rate” score as the total score.

### Quantitative Real-Time PCR

For validation, we did real-time reverse transcription-PCR (RT-PCR) for microarray experiment Real-time Reverse Transcription-PCR Analysis.

Real-time PCR was performed using the SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II (Tli RNaseH Plus) (RR820Q) according to the manufacturer’s instructions, in 96-well reaction plates. The forward primer sequences were USP15-F: 5’- TCAAAATGTG-TATCCTGGACCC-3’ and the reverse primer sequence was USP15-R: 5’-GTGCTATTGGCTCTTGACCTT-3’ and the product length was 246 bp. The primers of  $\beta$ -actin (reference gene): Human B-actin-F1: 5’-GAAGAGCTACGAGC-TGCCTGA-3’ and Human B-actin-R1: 5’- CAGACAG-CACTGTGTTGGCG-3’. Each sample was prepared in a total volume of 50  $\mu\text{l}$  containing 2  $\mu\text{l}$  of 0.4  $\mu\text{mol/l}$  primer mix, 25  $\mu\text{l}$  SYBR Green master mix, 4  $\mu\text{l}$  DNA template and 16  $\mu\text{l}$  RNase/DNase-free sterile water. The initial denaturation was carried out at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. The fluorescence data were collected in

the 60°C extension phase. Each sample was measured in 3 technical replicates.

### Cell Cultures

Human hepatocellular carcinoma cells SNU449 and HEP3B were cultured in DEME at 37 °C in a humidified incubator containing 5% CO<sub>2</sub>.

### Apoptosis and Proliferation Assay

Cells were harvest at logarithmic growth phase, then digested, resuspended with medium, adjust the cell density to 30000/ml. 100 µl of cell suspension were added to each well of a 96-well plate and incubate at 37° C; after 24 h, transfected with the corresponding siRNA; After 24 h, add 100 µL of staining solution (Hochest 10 µg/ml, PI 20 µM) to each well, and stain for 20 min at 37° C in the dark; open the Acumen power supply for preheating, open the apoptosis program for scanning, and output the results. For proliferation analysis, CCK8 was added to each well, incubated at 37° C for 1 h, and the absorbance was measured at 450 nm on an enzyme-linked immunosorbent assay.

### Statistical Analysis

The  $\Delta$ CT was used as an original interpretation of mRNA qPCR experiment based on the experimental data. The score  $\leq 3$  was regarded as high expression, while  $> 3$  was considered low expression. The differential expressions of USP15 in hepatocellular carcinoma tissues and adjacent tissues were evaluated by NPar Test. Survival curve depending on USP15 expression and clinical characters were drawing by Kaplan-Meier method and log-rank test. Subsequently, all the potential predict factors were involved in COX multivariate regression survival analysis. Spearman rank correlation coefficient was used to evaluate the correlation between USP15 expression and some clinical immunohistochemistry factors. All statistical analyses were conducted using SPSS 17.0 software.  $P < 0.05$  was considered significantly. The staining intensity (0/1+/2+/3+) and positive staining rate of the cytoplasmic staining/capsule staining of the antibody were read respectively.

## Results

### Increased Expression of USP15 With Disease Progression of Liver

Sporadic liver cancers are derived from sequential events. To exam the USP15 expression changes in the progression of liver diseases, we performed IHC analysis to test USP15 expression in a series of cancer-prone diseases including 2 normal liver tissues, 6 liver cirrhosis, 16 primary liver lesions and 15 metastases of hepatocellular carcinoma. The location of USP15 expression was mainly in cytoplasm and membrane (Figure 1 A-D). The cytoplasm USP15 expression was positively correlated with membrane USP15 expression ( $r = 0.680$ ,  $P = 0.004$ ).

The USP15 cytoplasm and membrane expression were lowest in normal liver and highest in metastatic foci of hepatocellular carcinoma (Figure 1E-F). The representative hepatocellular carcinoma cases with USP15 expression in tumor tissue were analyzed by IHC staining for USP15 (Figure 1 A-D).

### Association of USP15 With Survival in HCC

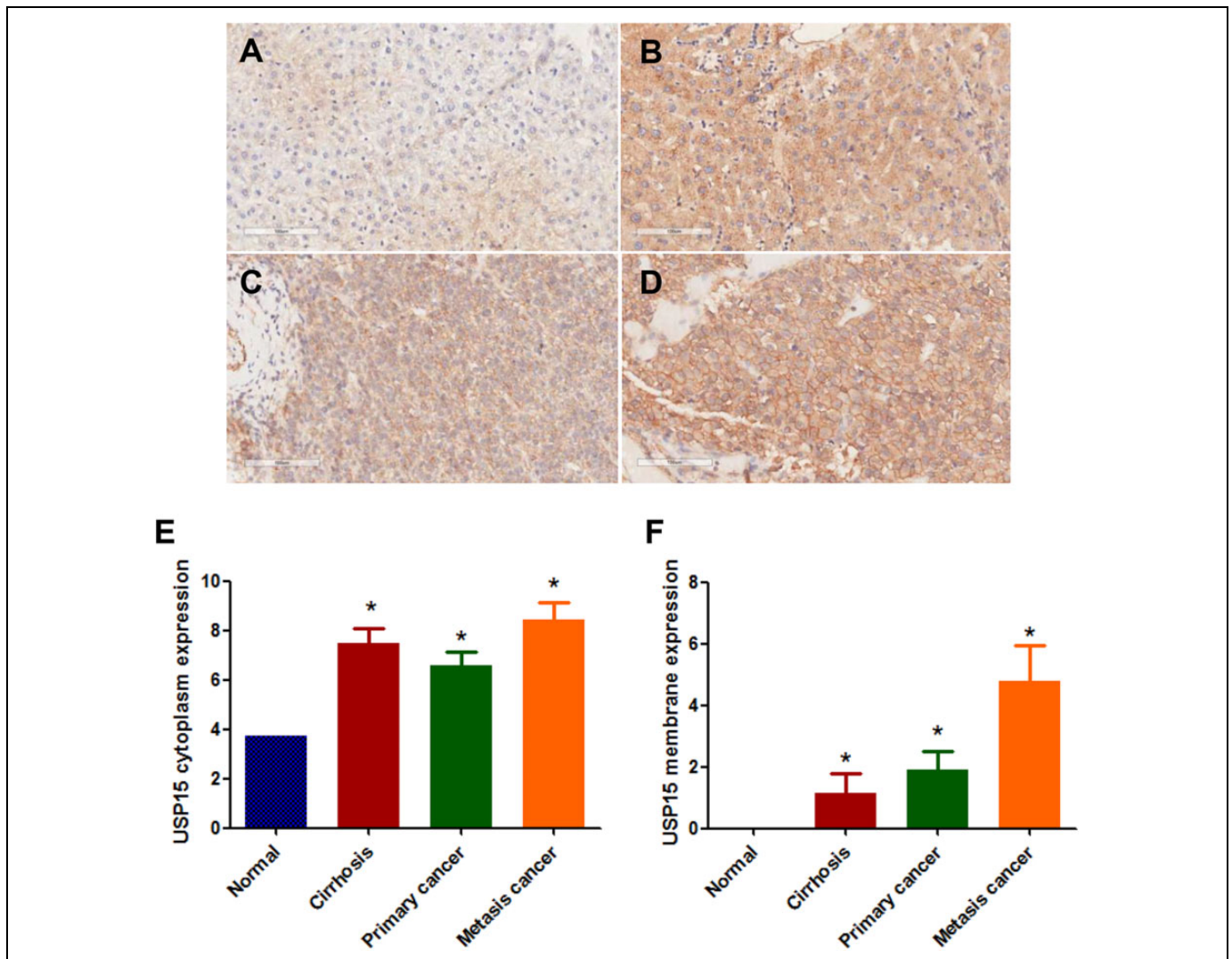
To identify the potential prognostic significance of USP15 in HCC, we perform qPCR on a cohort of 66 HCC tissues to value USP15 expression taking  $\beta$ -actin as referencing gene. The correlation between USP15 expression and hepatocellular carcinoma clinical parameters analyzed by the spearman's correlation analysis showed that the expression of USP15 in cancer tissues was positively correlated with the recurrence of hepatocellular carcinoma ( $r = 0.253$ ,  $P = 0.04$ ). The statistical analysis in detailed was shown in Table 2. The 66 HCC patients were cleaved into 2 groups according to different USP15 expression. HCC patients with high USP15 expression had a significantly worse prognosis in compared with those with low USP15 expression ( $P = 0.012$ ) (Figure 2). Subsequently, multivariate analyses of clinical factors associated with survival and USP15 expression in hepatocellular carcinoma were performed. USP15 expression was an independent predict factor ( $P = 0.001$ ) as well as tumor size (Table 3).

### USP15 Promoted Proliferation and Reduced Apoptosis of HCC Cell

USP15 expression was found to be commonly in various HCC cells (Figure 3). To test the USP15 function on HCC cells, we silenced USP15 in HCC cells and tested the influence on cell proliferation and apoptosis. The efficiency of USP15 inhibiting was detected by qPCR, and the results showed that the USP15 expression was efficiently silenced. Two different siUSP15, including siUSP15-1301 and siUP15-1535 transfection HEP3B cells reduced USP15 expression 56% and 24% respectively compared to negative control siRNA-NC. To investigate the effects of USP15 on HCC cells, siUSP15-1301 was transfected into SNU449 and Hep3B cells respectively. SNU449 and Hep3B cells with a siRNA-NC were taking as negative controls. Silencing of USP15 resulted in the proliferation rate of cells inhibited 28% and 43% in SNU449 and HEP3B cells respectively at 48 h compared to si RNA-NC group (Figure 4A), while induced increased the apoptosis rate of cells 246% and 69% respectively (Figure 4B).

## Discussion

Ubiquitin-specific proteases are reported associated with tumor progression. For example, USP7, USP17 and USP28 were highly expressed in lung cancer and colon cancer.<sup>8,9</sup> Overexpression of USP10, USP11, and USP22 in melanoma can be used as an indicator of clinical diagnosis and prognosis of tumors.<sup>10</sup> USP15, USP4 and USP10 were classified into the same subgroup of USPs. One assumption that USP15 function



**Figure 1.** Different USP15 expression in various liver diseases. Representative USP15 immunohistochemical staining figures in (A) normal liver tissues, (B) cirrhosis tissues, (C) primary cancer tissues and (D) metastasis site tissues. (E) Upregulated USP15 cytoplasm expression in liver diseases tissues. (F) Upregulated USP15 membrane expression in liver disease tissues. \* means  $P < 0.05$ .

**Table 2.** The Expression of USP15 in Cancer Tissues Was Positively Correlated With the Recurrence of Hepatocellular Carcinoma.

		Gender	Age	Tumor size	Tumor number	Pathology grade	T	N	M	Clinical stage	recurrence
USP15 expression	Correlation Coefficient	-.005	.210	.153	-.043	.036	.025	-.067	.229	.101	.253*
	Sig. (2-tailed)	.970	.090	.219	.732	.776	.839	.592	.065	.422	.040*
	N	66	66	66	66	66	66	66	66	66	66

\*. Correlation is significant at the 0.05 level (2-tailed).

similarly to USP4 is based on their similar structure. USP4 is extensively studied and considered to be an oncogene in liver cancer. Our experiment mainly studied the correlation between USP15 expression and hepatocellular carcinoma from mRNA and protein level.

In China, hepatocellular carcinoma has become a common malignant tumor due to its high morbidity and mortality,<sup>11</sup> and

the incidence of hepatocellular carcinoma showed significant regional differences with high incidence in Africa and East Asia. The overall prognosis of HCC patients is poor, with an overall 5-year survival rate of less than 20%.<sup>12</sup> The majority of hepatocellular carcinoma patients in China have chronic liver disease, and the onset of hepatocellular carcinoma is in the middle and late stage. To detect the characterization of the

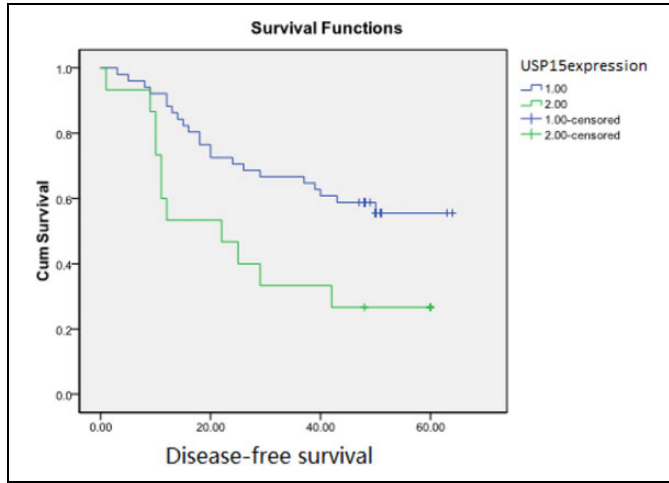
molecular mechanism and find independent predict factor of hepatocellular carcinoma have a significant sense on diagnosing and treating hepatocellular carcinoma.

Previous studied reported that USP15 predominantly localized in cytoplasm, and also take roles in nucleus and mitochondria, consistent with our immunohistochemistry results. The experiment results showed that the expression level of USP15 was highest in the hepatocellular carcinoma cell line

of SK-HEP-1, which was derived from ascites (hepatocellular carcinoma metastasis). The expression of USP15 was lowest in normal liver and highest in metastatic foci of hepatocellular carcinoma, which indicates that the expression of USP15 may be associated with inflammation and immune mechanisms that promote the metastasis of hepatocellular carcinoma. In recent years, USP15 has been reported to be involved in the regulation of a various of tumors. For example, USP15 deficiency in mice promotes antitumor T cell response to bacterial infection and tumor attack in vivo.<sup>13</sup> USP15 stabilizes TGF- receptor I and promotes tumorigenesis by activating signal transduction in glioblastoma.<sup>14</sup>

In addition, patients with high expression of USP15 have a worse prognosis in contrast with those with low USP15 expression. Moreover, the expression of USP15 in hepatocellular carcinoma tissue was significantly correlated with the recurrence of hepatocellular carcinoma. The expression of USP15 could be an independent predictor, and USP15 expression in cancer tissues were negatively correlated with the disease-free survival.

Silencing of USP15 promoted cell proliferation and enhanced cell invasion significantly. Previous reports revealed some target proteins of USP15, including p53 which had important relevance with apoptosis. Our results showed that silencing USP15 increased HCC cells apoptosis significantly. Many of the proposed USP15 targets participated in cancer-related progression, such as p53,<sup>15</sup> human homolog of mouse double minute 2,<sup>5</sup> the transforming growth factor  $\beta$  (TGF- $\beta$ ) receptor<sup>14</sup> and the ubiquitin (Ub) E3 ligase BRCA1-associated

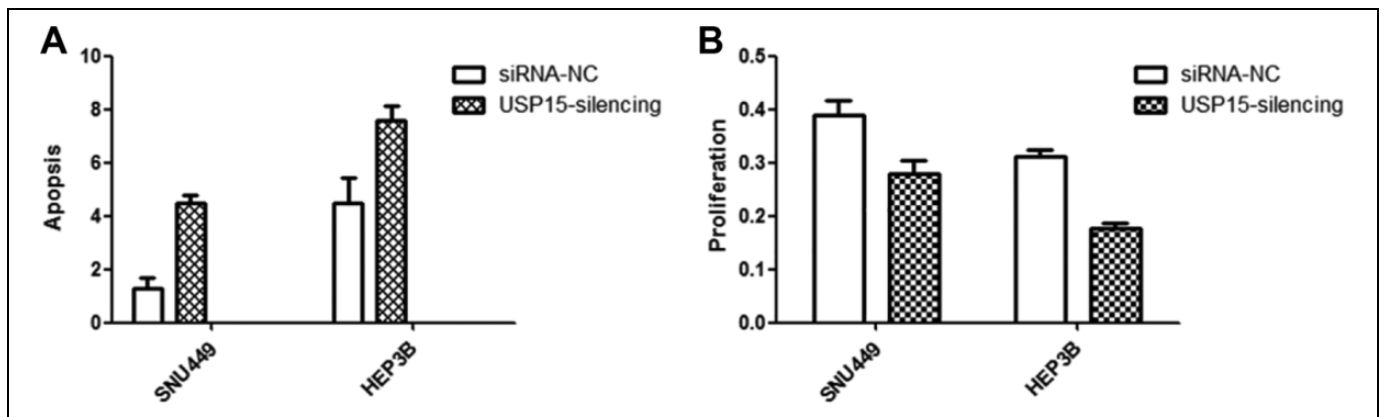


**Figure 2.** Association of USP15 with survival in HCC. High USP15 expression (green line 2.00) was associated with poor disease free survival in hepatocellular carcinoma (P = 0.012). P < 0.05 was considered statistically significant.

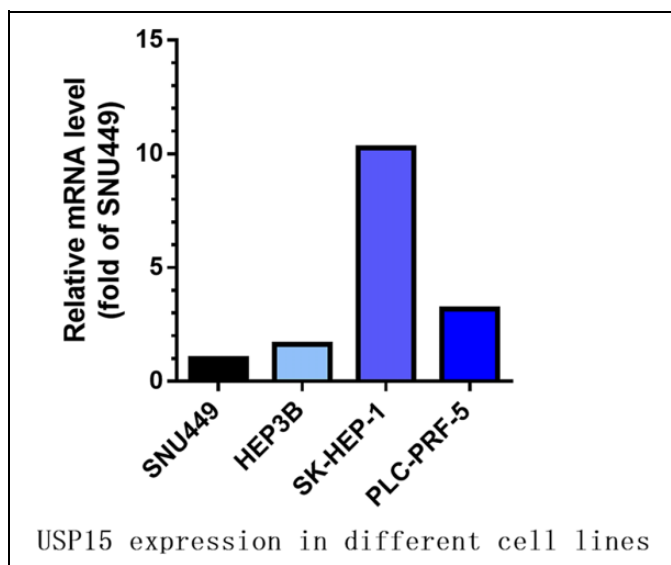
**Table 3.** COX Multivariate Regression Analysis of the Independent Predictors of USP15 in Hepatocellular Carcinoma Patients.

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
USP15 expression in cancer	1.004	.379	7.000	1	.008**	2.728	1.297	5.739
Gender	-1.734	1.034	2.812	1	.094	.177	.023	1.340
Tumor size	.765	.360	4.518	1	.034*	2.149	1.061	4.349

\*\* Correlation is significant at the 0.01 level (2-tailed). \* Correlation is significant at the 0.05 level (2-tailed).



**Figure 3.** USP15 commonly expressed in HCC cell lines.



**Figure 4.** USP15 promote apoptosis and inhibit proliferation of SNU449 and HEP3B.

protein associated with the Ras-MAPK signaling cascade.<sup>16</sup> The underlying mechanism of regulation of USP15 on cell apoptosis might be by regulating p53 activity which needs further research.

In conclusion, our experiments mainly studied the expression of USP15 in hepatocellular carcinoma, and it was proved that USP15 had a certain tumor promoting function in hepatocellular carcinoma. Therefore, we speculated that USP15 could be a potential target in hepatocellular carcinoma both in the level of mRNA and protein.

#### Author Contribution

Xue-Qing Yao and Ling Li are authors Equal contributors.

#### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.


#### Ethical Statement

This study was approved by the Shanghai outdo Ethical Committee (approval no. 81560392). All patients provided written informed consent prior to enrollment in the study.

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