# High expression of ubiquitin carboxyl-terminal hydrolase 22 is associated with poor prognosis in hepatitis B virus-associated liver cancer

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**Abstract.** Deubiquitinating enzymes regulate protein activity and cell homeostasis by removing ubiquitin moieties from various substrates. Ubiquitin carboxyl-terminal hydrolase 22 (USP22) is a member of the deubiquitinating protease family and is associated with the development of several tumor types. A previous study demonstrated that USP22 is highly expressed in liver cancer, and its high expression is associated with resistance to chemotherapy. However, the role of USP22 in hepatitis B virus (HBV)-associated liver cancer has not yet been elucidated. The current study demonstrated that USP22 was highly expressed in the tissues of patients with HBV-associated liver cancer, and its high expression was associated with clinicopathological characteristics, including tumor size, clinical stage and prognosis. Further results indicated that USP22 may regulate the proliferative and apoptotic abilities of HepG2.2.15 cells. Additionally, investigation into the underlying mechanism, using small interfering RNA, revealed that the downregulation of USP22 inhibited proliferation and promoted apoptosis though the phosphoinositide 3-kinase/protein kinase B signaling pathway. Therefore, USP22 has the potential to be used as an independent predictor of patient prognosis, as well as a therapeutic target for the treatment of HBV-associated liver cancer.

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*Key words:* ubiquitin carboxyl-terminal hydrolase 22, hepatitis B-associated liver cancer, phosphoinositide 3-kinase/protein kinase B, apoptosis, tumorigenesis

## Introduction

Liver cancer was reported in 2012 as the sixth most commonly diagnosed cancer, and the second most common cause of cancer-associated mortality worldwide (1). A total of 782,500 cases of liver cancer were diagnosed globally, resulting in 745,500 mortalities (1). China has a high incidence of liver cancer, accounting for ~50% of these cases (2). The main risk factors for the onset of liver cancer include chronic hepatitis B (CHB) virus (HBV) or hepatitis C virus infection, aflatoxin exposure, type II diabetes, liver cirrhosis and smoking (3-5). In China, 75-85% of cases are caused by CHB infection (6,7). A number of studies have reported that HBV may result in liver cancer development by interfering with several cellular processes, including transcription, signal transduction, cell cycle, protein breakdown, DNA repair, apoptosis and the maintenance of chromosome stability (8-10). Additionally, HBV may alter hepatocyte signaling and modulate the activity of transcription factors and target proteins involved in hepatocarcinogenesis (11). At present, the diagnosis and treatment of HBV-associated liver cancer remains inadequate. The majority of patients with HBV-associated liver cancer are diagnosed at intermediate or advanced stages (12), and the treatment is typically surgical resection. However, the aforementioned therapeutic strategy is unsatisfactory, and the survival rate remains low (13,14). Therefore, it is necessary to identify novel molecular markers for HBV-associated liver cancer and to elucidate their specific underlying molecular mechanisms, for improved clinical diagnosis and treatment.

Ubiquitin carboxyl-terminal hydrolase 22 (USP22) is a member of the deubiquitinating enzyme (DUB) family and is located on chromosome 17p11.2. The 1,578 bp USP22 open reading frame encodes 525 amino acids, containing highly conserved regions of the ubiquitin-specific-processing protease family (15). The USP22 protein is predominantly expressed in the cell nucleus and, through deubiquitinating modifications, exerts regulatory effects on cellular processes, including cell differentiation, cell cycle progression, transcriptional activation and signal transduction (16). Abnormally high USP22 expression has been detected in various cancer types, and is associated with tumor differentiation

and clinical prognosis (17-21). However, to the best of our knowledge, whether USP22 is involved in the development of HBV-associated liver cancer, and how it may regulate its development, have not yet been reported.

## Materials and methods

*Patients and samples*. Following formalin fixation, a total of 85 paraffin-embedded clinical liver tissue specimens (thickness 4 mm) from patients [n=85, range, 35-78 years of age, 1.2-20 cm of tumor size I-IV of Edmonson grade (22), I-IV of clinic stage (23), 3.7-8736 ng/ml of Alpha-fetoprotein] with liver cancer were obtained from the Department of Pathology of the Affiliated Hospital of Guilin Medical University (Guilin, China), and resection specimens (weight 2 g) from patients (n=85) with HBV-associated liver cancer were obtained following liver cancer resection surgery at this hospital between February 2013 and February 2017. Inclusion criteria were as follows: Comply with the diagnostic criteria for hepatitis B virus-associated liver cancer and all selected subjects were informed and agreed to the study. Exclusion criteria were as follows: Patients with non- hepatitis B virus-associated liver cancer; accompanied by any injury in the human body, including cardiovascular, liver, kidney, brain and blood system. The clinicopathological and survival data were collected and analyzed retrospectively. The present study was approved by the Ethics Committee of the Affiliated Hospital of Guilin Medical University. Written informed consent was obtained from all patients, according to the Declaration of Helsinki.

*Cell culture*. The human liver L02 cell line, as well as liver cancer HepG2.2.15, HuH7, HCCLM3 and Hep3B2.1-7 cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium containing 1% penicillin/streptomycin and 10% fetal bovine serum (all Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a 5% $CO_2$ incubator at 37˚C.

*Cell transfection*. The cells were transiently transfected when they became ~70%-80% confluent. Lipofectamine™ 3000 (5 $\mu$l; Invitrogen; Thermo Fisher Scientific, Inc.) was diluted in 125 $\mu$l opti-MEM (Gibco; Thermo Fisher Scientific, Inc.) medium and the USP22 small interfering (si)RNA or negative control siRNA (5 $\mu$l) was added to 125 $\mu$l opti-MEM medium and mixed for 5 min. The prepared Lipofectamine 3000 and siRNA dilutions were combined and gently mixed for 20 min. The six-well plates were removed from the incubator, washed 2-3 times with PBS, and 750 $\mu$l serum-free, antibiotic-free pure medium was added to each well. The Lipofectamine 3000-siRNA mixture was added to the cells, mixed and placed into the 5% $CO_2$ incubator at 37˚C. After 24 h of incubation at 37˚C, the following experiments were conducted. USP22 siRNA Sequence, sense CAGCAGCCC ACGGACAGUCUCAACA, anti-Sense UGUUGAGACUGU CCGUGGGCUGCUG.

*Cell proliferation measurement*. The Cell Counting Kit-8 (CCK8; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) assay was used to measure cell proliferation at 24, 48, 72 and 96 h. The cells were seeded in a 96-well culture plate (~$5x10^3$ cells/100 $\mu$l) following the addition of 10 $\mu$l CCK8 reagent to each well, and the plate was incubated in a $CO_2$ incubator at 37˚C for 1 h. The optical density value was measured at 450 nm using a microplate reader. The average value of each group was calculated and a proliferation curve was plotted.

*Apoptosis detection by flow cytometry*. Following digestion with 0.25% trypsin, cells were resuspended in PBS to prepare a single cell suspension with a density of ~$1x10^6$ cells/ml. The cell suspension from each group (100 $\mu$l) was stained with Annexin V-fluorescein isothiocyanate and propidium iodide using the Muse Annexin V and Dead Cell assay reagent (cat. no. MCH100105; Merck KGaA, Darmstadt, Germany) in equal proportions at room temperature in the dark for 20 min. Flow cytometry (Merck KGaA, Darmstadt, Germany) was used to determine the rate of apoptosis. Data were analyzed with FlowJo 10.0 software (FlowJo LLC, Ashland, OR, USA).

*Colony-formation assay*. Cells were seeded into a 6-well plate at $1x10^3$ cells/well, with three replicates for each group. After 2 weeks, the plate was removed from the incubator, washed three times with PBS, fixed in 4% paraformaldehyde at room temperature for 15 min and subsequently stained with 0.1% crystal violet at room temperature overnight. The plate was washed three times in PBS and cells were subsequently counted with the image J 1.48 software (National Institutes of Health, Bethesda, MD, USA), prior to statistical analysis of colony number.

*Immunohistochemistry*. The paraffin-embedded tissue sections (thickness, 3 mm) were deparaffinized in xylene at room temperature and rehydrated in a graded ethanol series. The sections were immersed in 3% hydrogen peroxide solution to inhibit endogenous peroxidase activity. Subsequent to being blocked in 5% non-fatty milk at room temperature in 1 h, they were subsequently incubated with the primary antibody (dilution, 1:200; Abcam; cat. no. 195289) for 1 h at 37˚C, and washed three times with PBS. An incubation with the secondary antibody using the MaxVision™ HRP-polymer anti-Rabbit IHC kit (cat. no. KIT-5006, Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China), according to the manufacturer's protocols, followed for 30 min at room temperature, and the sections were washed three times with PBS. The samples were stained with DAB for 5 min, counterstained with hematoxylin at room temperature and differentiated with 0.1% HCl alcohol for 1 sec. They were then dehydrated and dried in a graded alcohol series, hyalinized in xylene and sealed with neutral gum. The staining intensity score was calculated by using an Olympus X71 inverted microscope (magnification, x200; Olympus Corp., Tokyo, Japan) as follows: 0, no staining; 1+, mild staining; 2+, moderate staining; 3+, heavy staining. The staining area scores were as follows: 0, no staining under the microscope; 1+, positive staining of <30% of tissue; 2+, positive staining of 30-60% of tissue; 3+, positive staining of >60% of tissue. The sum of the staining intensity and area scores was used to evaluate the expression of USP22, with the highest score being 6 and the lowest being 0. The criterion for positive expression of USP22 was the presence of brown staining in the nucleus.

*RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).* TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) reagent was added to 0.1 g tissue, which was subsequently cut into pieces. Chloroform (200 $\mu$l) was added and the mixture was centrifuged in 13,523 x g at 4˚C for 15 min. A total of 400 $\mu$l upper layer liquid was collected and mixed with an equal volume of isopropanol, prior to further centrifugation in 13,523 x g at 4˚C for 15 min. The supernatant was subsequently removed and 1 ml 70% ice-cold alcohol was added to wash the RNA. Following centrifugation in 1,127 x g at 4˚C for 5 min, the supernatant was removed, and the RNA was dried and reconstituted in diethyl pyrocarbonate-treated water. The product was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.), and 500 ng was reverse transcribed using the QuantiTect Reverse Transcription kit (Qiagen, Inc., Valencia, CA, USA). The reaction cycling conditions were performed as follows: 1 cycle at 95˚C for 15 min, followed by 40 cycles at 95˚C for 10 sec and at 60˚C for 60 sec. The ratio between the target gene and β-actin was used to calculate the relative quantitation. The amplification products and the relative expression of RT-PCR were analyzed using the ΔΔCq method (24). β-actin was used as an internal control to normalize gene expression levels. The primers used for amplification were as follows: β-actin, forward primer, 5'-AAGGAAGGCTGGAAGAGTGC-3', reverse primer, 5'-CTGGGACGACATGGAGAAAA-3'; USP22, forward primer, 5'-GGCGGAAGATCACCACGTAT-3', reverse primer, 5'-TTGTTGAGACTGTCCGTGGG-3'.

*Western blot analysis.* Samples were collected and lysis buffer [137 mM NaCl, 50 mM Tris-HCl, 10% glycerol, 100 mM sodium orthovanadate, 10 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF),1% Nonidet P-40,10 mg/ml leupeptin, and 5 mM protease inhibitor cocktail; pH 7.4] was added to extract total proteins., and the concentration was determined using the bicinchoninic acid method. The proteins (30 ug) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes by wet transfer. The membranes were blocked in 5% skimmed milk/ tris-buffered saline with Tween-20 (TBST) for 1 h at 37˚C. The membrane was washed three times in TBST. Membranes were subsequently incubated with primary antibody overnight at 4˚C. On the following day, the membrane was washed three times with TBST, and then incubated with secondary antibody (HRP-labeled goat anti-rabbit IgG; cat. no. A0208; Beyotime Institute of Biotechnolgy) at a 1:10,000 for 1 h at 37˚C. Subsequently, chemiluminescence was used to display the imprinting, and the results were analyzed using the Tannon 5200 chemiluminescent imaging system (Tanon Science and Technology, Shanghai, China). The primary antibodies of USP22 (abcam195289, concentration is 1:1,000), β-actin, activated caspase-3 (abcam136812, concentration is 1:1,000), caspase-8 (abcam25901, concentration is 1:1,000), and caspase-9 (abcam2324, concentration is 1:1,000). The primary antibodies of phosphoinositide 3-kinase (PI3K) (SC-136298), phosphorylated (p)-PI3K (Cat. No. 20584-1-AP), protein kinase B (Akt) (SC-135829) and p-Akt (SC-271964) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and proteintech group (Wuhan, China). Protein bands were visualized by Image J2x (National Institutes of Health, Bethesda, MD, USA).

*Microarray analysis.* Total RNA quality and quantity were determined using NanoDrop ND-2000 (Thermo Fisher Scientific, Inc.). GeneChip Human Genome HU U133 plus 2.0 arrays (Affymetrix; Thermo Fisher Scientific, Inc.) were used according to the manufacturer's protocol. The data were initially normalized by robust multiarray average normalization algorithms in Transcriptome Analysis Console 4.0 software (Affymetrix; Thermo Fisher Scientific, Inc.). Significantly altered genes between USP22 knockdown and its control cells were visualized by scatter plots. Clustering analysis was performed using Gene Cluster v3.0 software. Gene set enrichment analysis was conducted using ConceptGen (National Center for Integrative Biomedical Informatics, Ann Arbor, MI, USA).

*Statistical analysis.* SPSS v22.0 software (IBM Corp., Armonk, NY, USA) was used to analyze the experimental data. The $\chi^2$ test was used to evaluate the association between USP22 expression and clinicopathological parameters. Multigroup comparisons of means were performed by one-way analysis of variance test with post-hoc comparisons using the Student-Newman-Keuls test. Receiver operating characteristic curve analysis overall survival (OS) time and relapse-free survival (RFS) time were also analyzed in the present study. Student's t-test was used to analyze continuous variables and the Kaplan-Meier method was used to assess survival probabilities. $P<0.05$ was considered to indicate a statistically significant difference. Each group of experiments was repeated three times.

## Results

*USP22 is highly expressed in HBV-associated liver cancer tissues.* The RT-qPCR results demonstrated that in the 10 normal liver, 15 liver with CHB infection, 23 non-HBV-associated liver cancer and 28 HBV-associated liver cancer tissues, the expression levels of USP22 exhibited a gradual increase. The expression of USP22 in tissues of HBV-associated liver cancer was higher than in that measured in normal liver, liver with CHB infection, and non-HBV-associated liver cancer ($P<0.01$, $P<0.01$ and $P<0.05$, respectively; Fig. 1A). Furthermore, 18 pairs of carcinoma and para-carcinoma tissues from patients with HBV-associated liver cancer were randomly selected to analyze the protein expression of USP22 by western blot analysis (Fig. 1B). It was revealed that the expression level of USP22 in carcinoma tissues was significantly increased compared with the para-carcinoma tissues ($P<0.01$; Fig. 1C). Immunohistochemistry was subsequently performed to detect USP22 expression in 85 paraffin-embedded sections of HBV-associated liver cancer tumors (Fig. 1D). The results revealed that positive USP22 protein staining was predominantly located in the nucleus, and was more highly expressed in the cancer tissues compared with the para-carcinoma tissues ($P<0.001$; Fig. 1E).

*USP22 is associated with poor clinical prognosis in patients with HBV-associated liver cancer.* The expression levels of USP22 in HBV-associated liver cancer were divided into

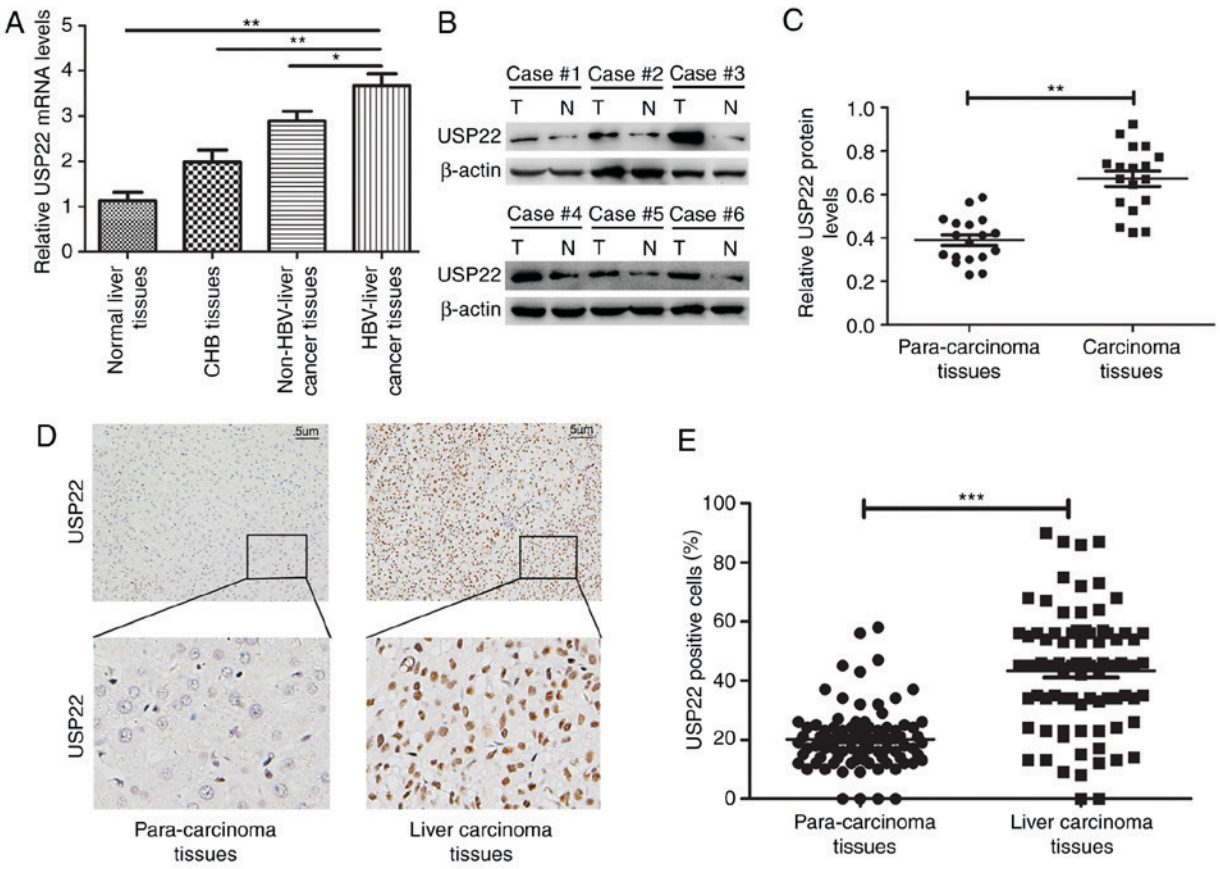


Figure 1. USP22 is highly expressed in HBV-associated liver cancer tissues. (A) Relative USP22 mRNA expression was determined in tissue from 10 normal liver, 15 CHB liver, 23 non-HBV-associated liver cancer and 28 HBV-associated liver cancer samples. (B) Western blot images and (C) relative quantification of USP22 protein expression in 18 cases of HBV-associated liver cancer carcinoma and para-carcinoma tissues. (D) Representative images and (E) expression levels of USP22 protein in 85 cases of HBV-associated liver cancer carcinoma tissues and para-carcinoma tissues as detected by immunohistochemistry. $^{*}$P<0.05, $^{**}$P<0.01 and $^{***}$P<0.001. Error bars represent the standard deviation. USP22, ubiquitin carboxyl-terminal hydrolase 22; HBV, hepatitis B virus; CHB, chronic hepatitis B; T, tumor; N, non-tumor.

high- (n=45) and low-expression (n=40) groups, according to the immunohistochemical scoring system described above, and compared against clinicopathological data (Table I). Statistical analysis determined that the expression of USP22 was associated with tumor size (P<0.01), Edmonson grade (P<0.01), clinical stage (clinical staged according to the pTNM (pathologic tumor, node, metastasis) classification criteria of the International Union Against Cancer) (P<0.01) and number of tumors (P<0.05). Receiver operating characteristic curve analysis revealed that USP22 expression was statistically significant in terms of overall survival (OS) time (P<0.01; Fig. 2A) and recurrence -free survival (RFS) time (P<0.01; Fig. 2B). The association between USP22 expression and the clinical prognosis of patients with HBV-associated liver cancer was further analyzed using Kaplan-Meier survival curves. The results revealed that the OS time of the USP22 high-expression group was lower than that of the USP22 low-expression group (P<0.01; Fig. 2C), as was the RFS time (P<0.01; Fig. 2D). Together, the results demonstrate that high USP22 expression in HBV-associated liver cancer is associated with the degree of malignancy and poor clinical prognosis of patients.

*USP22 is highly expressed in HepG2.2.15 cells, and USP22-siRNA effectively silences the expression of USP22.* Normal liver L02 cells and 4 liver cancer cell lines (Hep3B, Huh7, HCCLM3 and HepG2.2.15) were used to explore USP22 protein expression by western blot analysis. The USP22 protein levels in the liver cancer cells was higher than that in the normal liver cells, and the highest was measured in HepG2.2.15 cells (Fig. 3A and B). USP22-siRNA was transiently transfected in HepG2.2.15 cells to silence USP22 expression, and the transfection efficiency was confirmed by western blotting. The results revealed that USP22-siRNA effectively decreased USP22 expression (P<0.01 versus control; Fig. 3C and D).

*Downregulation of USP22 inhibits the proliferative ability of HepG2.2.15 cells and promotes apoptosis.* Following knockdown of USP22 expression with siRNA, the proliferative ability of HepG2.2.15 cells was tested with a CCK8 assay and colony-formation experiments. The results revealed that silencing USP22 significantly inhibited HepG2.2.15 cell proliferation at 96 h (P<0.01; Fig. 4A). The colony-formation experiments demonstrated that HepG2.2.15 cells in the USP22-siRNA group formed significantly fewer colonies compared with the control groups (P<0.01; Fig. 4B). Flow cytometry was subsequently used to detect cell apoptosis, and the results revealed that the apoptotic rate of HepG2.2.15 cells was significantly increased following USP22 silencing (P<0.01; Fig. 4C and D). Furthermore, western blot analysis determined that the expression of apoptosis-associated proteins, cleaved

Table I. Association between USP22 staining results and clinicopathological characteristics of 85 patients with liver cancer.

| | USP22 staining | | | |
|---|---|---|---|---|
| Variables | Low | High | Total | P-value |
| Age, years | | | | 0.32 |
| <50 | 17 | 24 | 41 | |
| ≥50 | 23 | 21 | 44 | |
| Sex | | | | 0.18 |
| Male | 35 | 43 | 78 | |
| Female | 5 | 2 | 7 | |
| AFP, ng/ml | | | | 0.06 |
| <200 | 26 | 20 | 46 | |
| ≥200 | 14 | 25 | 39 | |
| Tumor size (maximum diameter), cm | | | | <0.01 |
| <5 | 25 | 12 | 37 | |
| ≥5 | 15 | 33 | 48 | |
| Edmonson grade (22) | | | | <0.01 |
| I+II | 34 | 26 | 60 | |
| III+IV | 6 | 19 | 25 | |
| Clinical stage (23) | | | | <0.01 |
| I+II | 21 | 5 | 26 | |
| III+IV | 19 | 40 | 59 | |
| Tumor number | | | | <0.05 |
| 1 | 28 | 21 | 49 | |
| >1 | 12 | 24 | 36 | |

USP22, ubiquitin carboxyl-terminal hydrolase 22; AFP, Alpha-fetoprotein.

caspase-3 and -9 increased in the USP22-siRNA group compared with the control (P<0.01), whereas no difference was observes in the levels of cleaved caspase-8 (Fig. 4E and F).

*Silencing USP22 inhibits PI3K/Akt protein expression*. In order to further clarify the specific molecular mechanism of USP22 in liver cancer, microarray analysis was performed to determine the expression profiles of HepG2.2.15-USP22-siRNA and HepG2.2.15 control cells. Following USP22 knockdown, a series of signaling pathways were significantly changed; the PI3K/Akt signaling pathway was the most enriched apoptotic pathway (Fig. 5A). The results of the microarray analysis were verified by western blot analysis, which revealed that the levels of PI3K, Akt were significantly decreased (P<0.05; Fig. 5B and C).

## Discussion

HBV-associated liver cancer is one of the most common types of liver cancer, particularly in China (8). Due to rapid tumor progression, it is difficult to provide an early diagnosis and the associated mortality rate is high (8). At present, HBV-associated liver cancer remains a focus of research for identifying the key regulatory factors controlling the development and progression of this disease, in order to improve clinical diagnosis and treatment. Ubiquitination and deubiquitination are important post-translational modifications that participate in a variety of biological behaviors (25,26). As an important member of the DUB family, and as the enzymatic center of the deubiquitinating module, USP22 participates in a series of biochemical reactions (16). Studies have revealed that removal of USP22 leads to a decrease, rather than an increase, in global monoubiquitination of histone H2B (H2Bub1). By contrast, depletion of non-enzymatic components ataxin-7-like protein 3 (ATXN7L3) or transcription and mRNA export factor ENY2, results in increased H2Bub1 levels. Two new H2Bub1 DUBs, USP27X and USP51, which function independently of the Spt-Ada-Gcn5 acetyltransferase complex, compete with USP22 for ATXN7L3 and ENY2 for activity (27). Inhibition of these DUBs suppresses tumor growth, and this has become a focus of tumor marker research.

A previous study demonstrated that USP22 was highly expressed in liver cancer tissue and was associated with the occurrence and development of liver cancer, and poor prognosis (28). Silencing of USP22 inhibited the cell proliferation, migration and invasion, and the chemotherapeutic resistance of liver cancer cells. In addition, it was demonstrated that USP22 is an independent biomarker for the prediction of survival and prognosis for patients with liver cancer (28). The focus of the present study was HBV-associated liver cancer; the association between this cancer type and USP22 was verified, and the potential underlying regulatory mechanism was explored. The

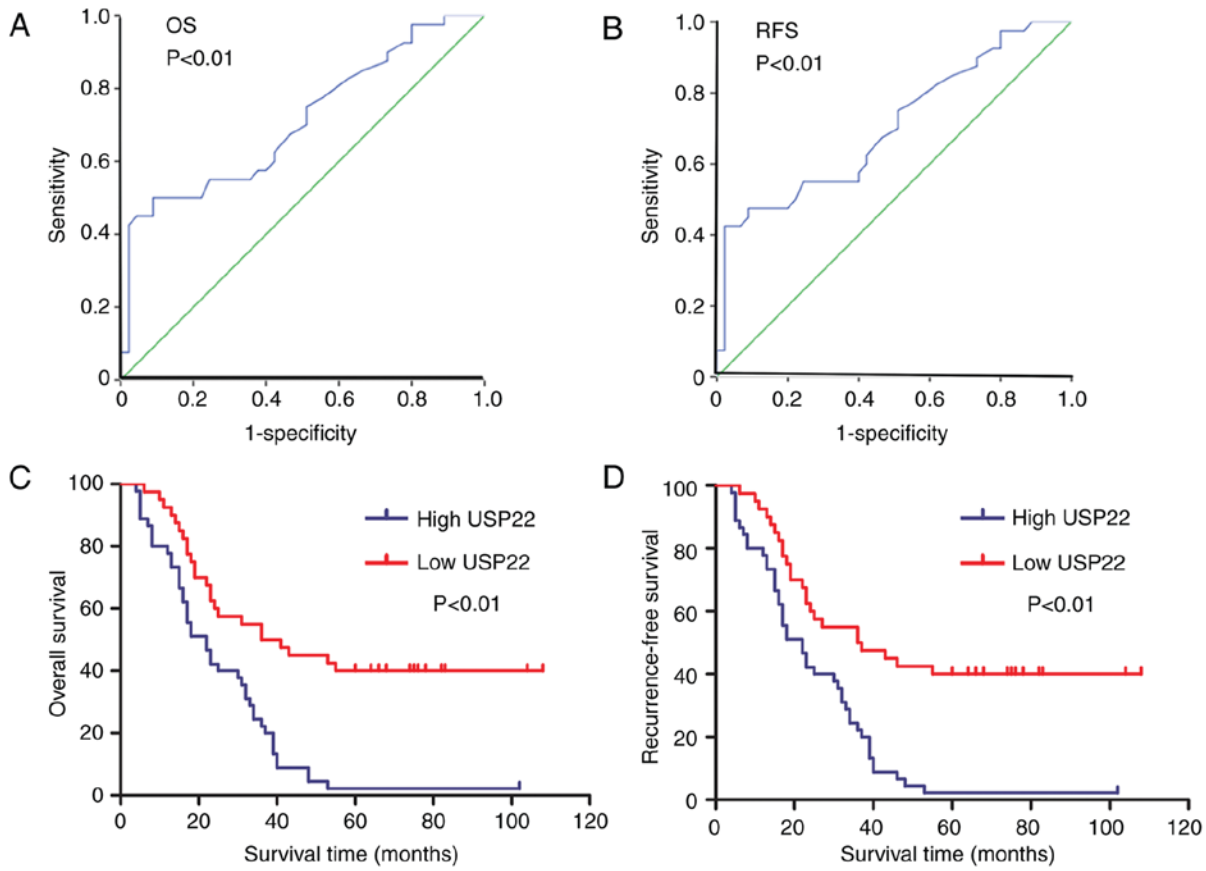


Figure 2. USP22 is associated with poor clinical prognosis in patients with HBV-associated liver cancer. Receiver operating characteristic curve analysis was performed to determine the significance of USP22 protein expression on the (A) OS and (B) RFS time of patients with HBV-associated liver cancer. Kaplan-Meier analysis of the relationship between USP22 protein expression and patient (C) OS and (D) RFS times. USP22, ubiquitin carboxyl-terminal hydrolase 22; HBV, hepatitis B virus; OS, overall survival; RFS, recurrence-free survival.

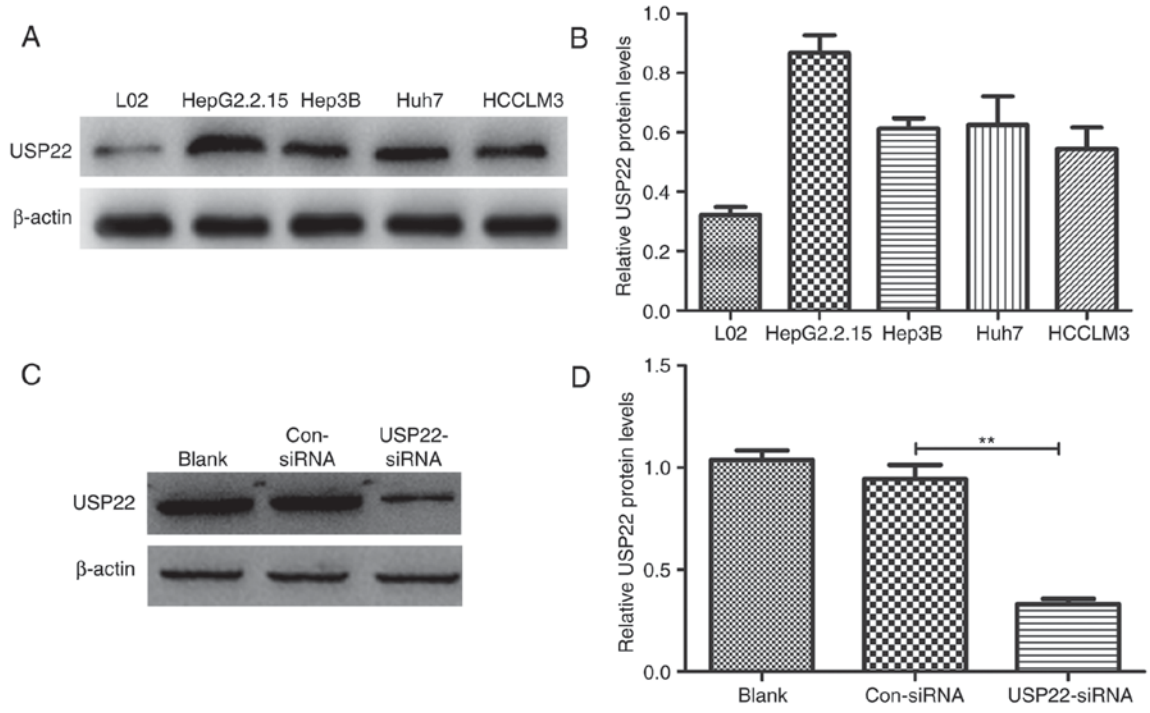


Figure 3. USP22 is highly expressed in HepG2.2.15 cells, and USP22-siRNA effectively silences the expression of USP22. (A) Western blotting and (B) relative quantification of USP22 protein expression in the normal liver cell line LO2 and four liver cancer cell lines (Hep3B, Huh7, HCCLM3 and HepG2.2.15). (C) Western blotting and (D) relative quantification of USP22 protein expression following USP22-siRNA transfection. $^{**}$P<0.01. Error lines indicate the standard deviation. The experiments were repeated three times. USP22, ubiquitin carboxyl-terminal hydrolase 22; siRNA, small interfering RNA; Con, control.

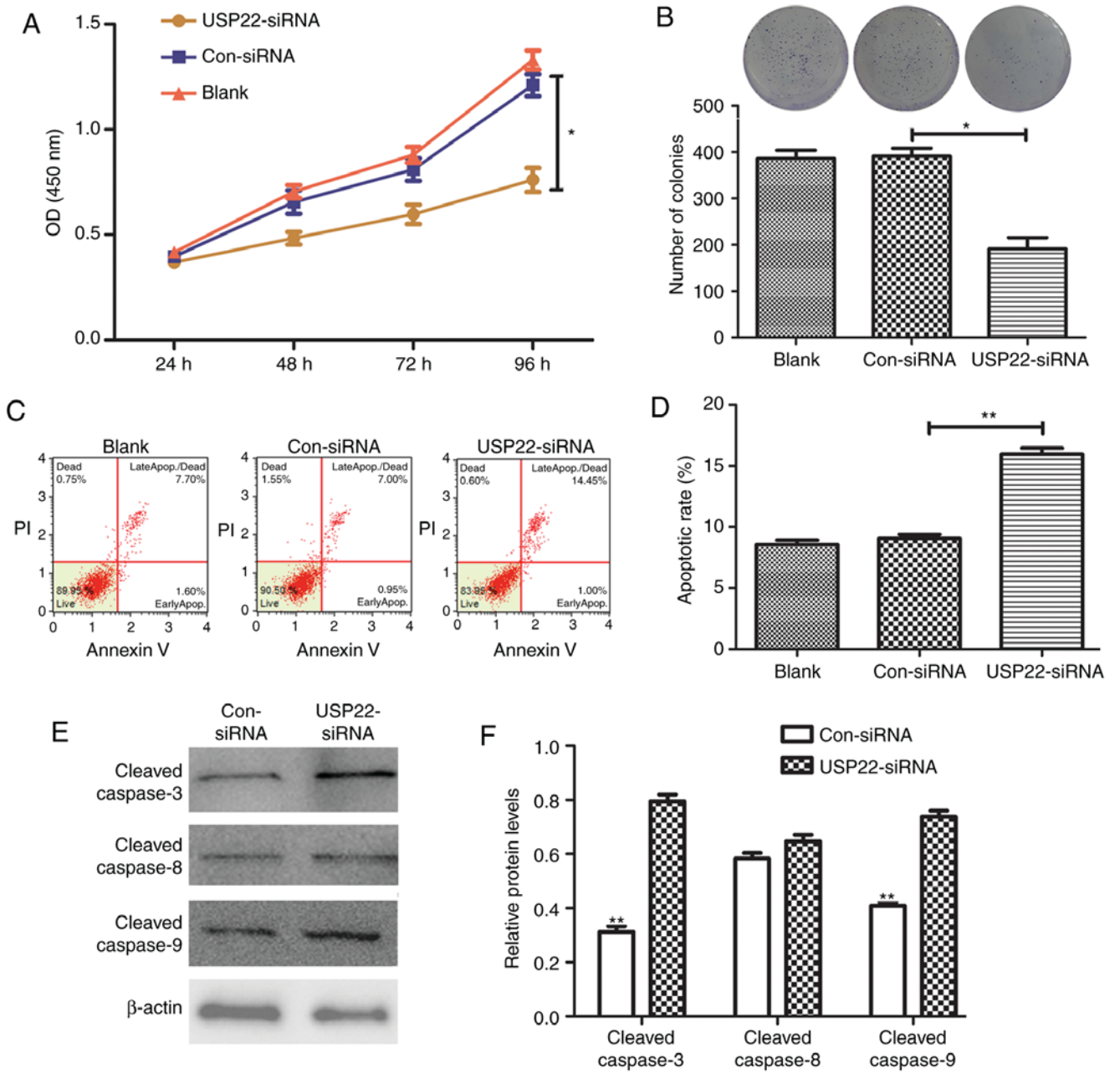


Figure 4. Downregulation of USP22 inhibits HepG2.2.15 cell proliferation and promotes apoptosis. The effect of USP22 downregulation on HepG2.2.15 cell proliferation was determined with (A) the Cell Counting Kit-8 and (B) a colony-formation assay. *P<0.05. (C) Apoptosis was analyzed by annexin V-fluorescein isothiocyanate/PI flow cytometry and (D) the results were quantified. **P<0.01. (E) Western blotting and (F) relative quantification of cleaved caspase-3, -8 and -9 proteins. **P<0.01 versus the equivalent USP-siRNA group. The experiments were repeated three times. USP22, ubiquitin carboxyl-terminal hydrolase 22; OD, optical density; siRNA, small interfering RNA; Con, control; PI, propidium iodide.

results revealed that USP22 expression was varied among normal liver, liver with CHB infection, non-HBV-associated liver cancer and HBV-associated liver cancer tissues. However, the levels were significantly higher in HBV-associated liver cancer tissues, and high expression was demonstrated to be associated with tumor development and poor prognosis.

In the *in vitro* experiments, the expression of USP22 was higher in liver cancer cells compared with normal liver cells, and HepG2.2.15 cells exhibited the highest expression of USP22 among the liver cancer cell lines. Silencing of USP22 inhibited proliferation, promoted apoptosis and increased the activation levels of the apoptosis-associated proteins caspase-3 and -9 in HepG2.2.15 cells.

Numerous studies have confirmed that USP22 affects the expression of its target genes, including Myc proto-oncogene protein (29), polycomb complex protein BMI-1 (30), fructose-1,6-bisphosphatase 1 (31), focal adhesion kinase 1 (32) and transforming growth factor β (33), by removing ubiquitin ligase from protein substrates, thereby regulating a series of biological behaviors, including cell cycle progression, proliferation and differentiation, and epithelial to mesenchymal transition. As a major signal transduction pathways, PI3K/Akt signaling inhibits apoptosis and promotes cell proliferation by influencing the activation state of a variety of downstream molecules. It has been demonstrated that the PI3K/Akt/mammalian target of rapamycin signal transduction

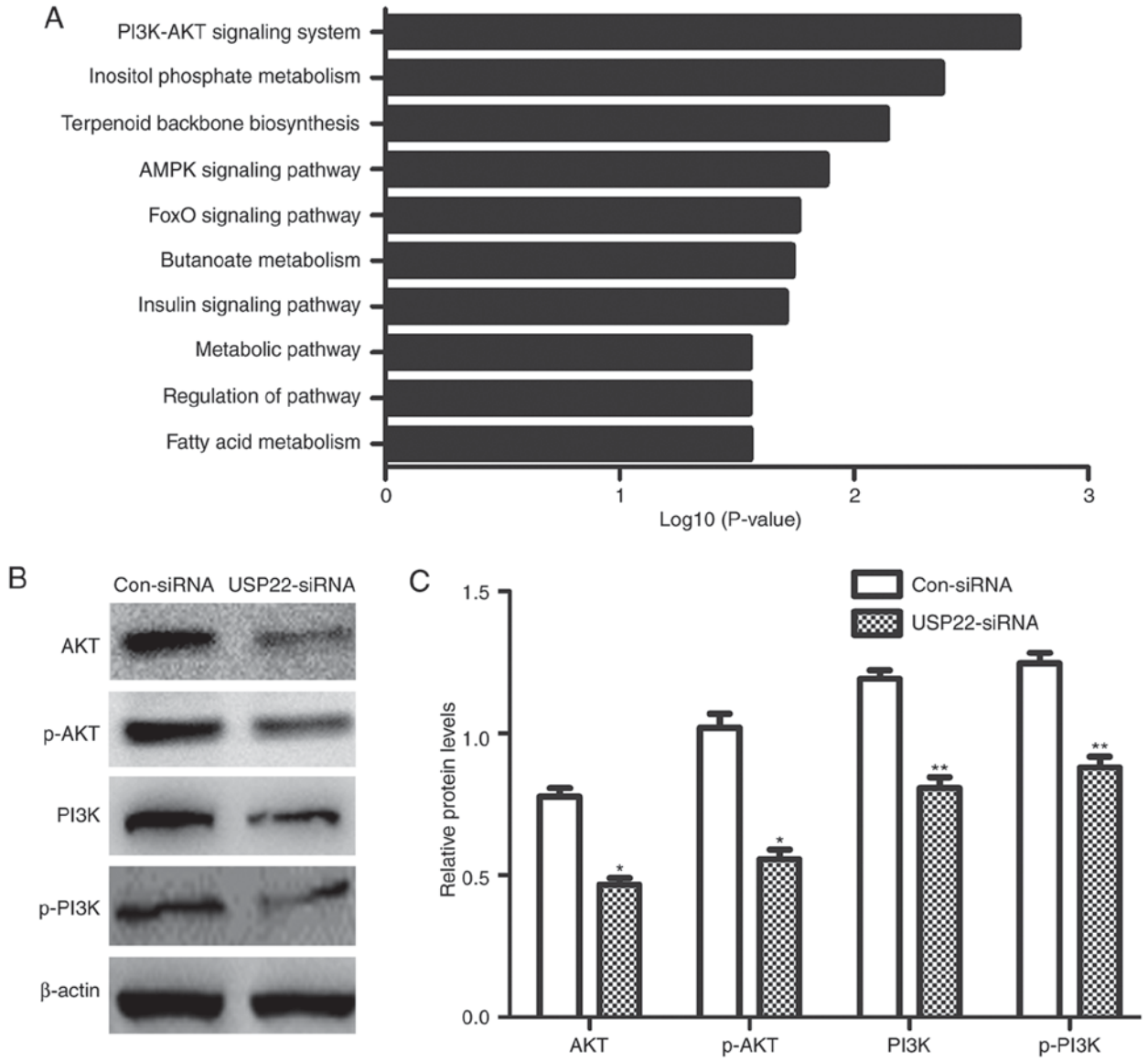


Figure 5. USP22 silencing inhibits PI3K and Akt protein expression. (A) Results of the microarray analysis. (B) Western blotting and (C) relative quantification of PI3K, p-PI3K, Akt and p-Akt proteins. $^{*}$P<0.05 and $^{**}$P<0.01 versus the equivalent Con-siRNA group. The experiments were repeated three times. USP22, ubiquitin carboxyl-terminal hydrolase 22; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; AMPK, AMP-activated protein kinase; FOXO, Forkhead box protein O; p-, phosphorylated; siRNA, small interfering RNA; Con, control.

pathway serves a critical role in tumorigenesis and development. Therefore, it has become a potential novel target for tumor treatment (21). It has been reported that HBV X protein promotes the malignant transformation of hepatocytes, by driving the expression of α-fetoprotein to activate the PI3K/Akt signaling pathway, which in turn stimulates the expression of reprogramming-associated proteins and oncogenes (34). HBV large surface proteins are involved in the development of liver cancer by activating the proto-oncogene tyrosine-protein kinase Src/PI3K/Akt signaling pathway and accelerating G1/S cell cycle progression (35). In addition, studies have reported that serine/threonine-protein kinase PAK1 interacts with the PI3K/Akt signaling pathway to promote the proliferation and migration of liver cancer cells (36). In the present study, it was demonstrated that silencing USP22 in HepG2.2.15 cells modulated the expression of key proteins in PI3K/Akt pathway, and decreased the levels of PI3K, Akt,. Therefore, it could be concluded that USP22 may serve an important role in inducing apoptosis and inhibiting proliferation of liver cancer cells through mechanisms affecting PI3K/Akt expression levels. In conclusion, it was determined that USP22 was highly expressed in HBV-associated liver cancer tissues and was associated with tumor differentiation and poor prognosis. In addition, it was revealed that USP22 regulated the proliferation and apoptosis of HepG2.2.15 cells. In terms of molecular mechanism, microarray and western blot analysis verified that USP22 regulated the expression of PI3K/Akt pathway-associated proteins, and therefore may regulate hepatocyte apoptosis. Our next aim is to further clarify which specific PI3K/Akt signaling molecules are affected by USP22, by investigating the association between USP22 knockdown and the Akt pathway by means of small molecules that activate Akt signaling. The present results suggest that USP22 may be used as an independent predictor of patient survival and

prognosis, as well as a potential molecular target for the treatment of HBV-associated liver cancer.

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**Availability of data and materials**

The datasets used during the present study are available from the corresponding authors upon reasonable request.

**Authors' contributions**

BT and ZW conceived and designed the experiments. YL performed the experiments. XL, WL, ZL, YW, LW and SZ analyzed the data. BT supervised the experiments and revised the manuscript. WJL and YL wrote the manuscript. All authors read and approved the manuscript.

**Ethics approval and consent to participate**

The present study was approved by the Ethics Committee of Guilin Medical University (Guilin, China). Written informed consent was obtained from all patients.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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