



## KNOCK-DOWN OF USP22 BY SMALL INTERFERING RNA INTERFERENCE INHIBITS HEPG2 CELL PROLIFERATION AND INDUCES CELL CYCLE ARREST

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### Abstract

The Ubiquitin-specific protease 22 (USP22) is a new putative cancer stem cell marker, which plays a significant role in tumorigenesis and cell-cycle progression. However, little is known about the impact of USP22 knock-down on the growth of human hepatoma cell lines. In this study, elevated expression of USP22 was observed in the human HepG2 hepatic cancer cell line compared to the normal human hepatocyte Chang liver cell line. Subsequently, we used siRNA specifically suppressing expression of USP22 and observed that the knock-down of USP22 could effectively induce cell cycle arrest and inhibit HepG2 cell proliferation. Furthermore, our results showed that USP22 deletion caused down-regulation of cyclin D2 expression and up-regulation of p15 and p21 expression. Collectively, Our findings indicate that USP22 may be responsible for HepG2 cell growth and USP22 regulates the cell cycle via the c-Myc/cyclin D2 pathway and down-regulating p15 and p21 expression in HepG2 cell.

**Key words:** USP22, Liver Neoplasms, Small Interfering RNA (siRNA), Cell Cycle.

### Article information

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignant tumor worldwide and causes approximately 600,000 deaths globally each year (9). Despite improvements in clinical treatments, such as surgical resection, liver transplantation and interventional therapy, HCC prognosis is still very poor. Because tumorigenesis and tumor progression in hepatic cells are the results of multiple genetic alterations, a single molecule targeting therapy has yet to be discovered. Thus, identification of target molecules that control the biological characteristics of HCC is of great importance.

Recent observations have identified an 11-gene Polycomb/cancer stem cell signature that could predict the likelihood of treatment failure in cancer patients (2). Ubiquitin-specific protease 22 (USP22) is a new putative cancer stem cell marker involved in the 11-gene Polycomb/cancer stem cell signature (14,15). USP22 belongs to a large family of proteins with ubiquitin hydrolase activity. USP22 is described as a key subunit of the human Spt-Ada-Gcn5 acetyltransferase (hSAGA) transcriptional cofactor complex, which is required for activator-driven transcription and cell cycle progression (15). Within hSAGA, USP22 removes ubiquitin from histone H2B, thus regulating the transcription of downstream genes related to epigenetic alteration and cancer progression. Moreover, USP22 is required for the proper functioning of MYC, which is widely believed to play a significant role in regulating the tumor cell cycle and tumor invasion (14,15). More recent studies have demonstrated that USP22 can inhibit the transcription of the p21 gene by deubiquitinating the transcriptional regulator FBP1, leading to cell proliferation and tumorigenesis (1).

Little is known about the impact of USP22 knock-down on the growth of human hepatoma cell lines. In this study, we used siRNA specifically suppressing expression of

USP22 and observed that the knock-down of USP22 could effectively inhibit HepG2 cell proliferation and induce cell cycle arrest. We also suggest that USP22 may regulate cell cycle progression by down-regulating p15 and p21 expression and up-regulating cyclin D2 expression.

## MATERIALS AND METHODS

### Cell culture antibodies and reagents

The human HepG2 hepatic cancer cell line was purchased from American Type Culture Collection (ATCC). The cell line cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS, Gibco, US) and 100 µg/ml each of penicillin and streptomycin (Invitrogen, US) in 5% CO<sub>2</sub> at 37°C. The USP22 antibody (ab4812, 1:500) was from Abcam (UK). β-Actin (TA-09, 1:500) and p21 (ZS6246, 1:200) antibodies were from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (China). The Cyclin D2 (bs-1148R, 1:200) antibody was from Beijing Biosynthesis Biotechnology Co., Ltd. (China). The p15 (INK4B/CDKN2B)(KG2261, 1:500) antibody was from KeyGen BioTECH (China). Lipofectamine 2000 was from Invitrogen Corp. (USA). The reverse-transcription polymerase chain reaction (RT-PCR) kit and primers were from Takara (Japan). The MTT assay kit was from Sigma. The Annexin V-FITC Apoptosis Detection Kit (KGA106) and the Cell Cycle Detection Kit (KGA511) were from KeyGen BioTECH (China).

### RNA extraction and RT-PCR

Total-RNA was prepared using the RNAiso™ Plus kit (Takara), and the concentration of RNA was evaluated with a spectrophotometer. The primers for amplification were as follows: USP22, forward primer, 5'-GGACAACTG-GAAGCAGAACC-3' and reverse primer, 5'-TGAAA-CAGCCGAAGAAGACA-3; β-actin, forward primer, 5'-AAGGAAGGCTGGAAGAGTGC-3' and reverse

primer, 5'-CTGGGACGACATGGAGAAAA-3'. RT-PCR reactions were performed using the Takara RNA PCR kit (AMV) version 3.0 according to the manufacturer's protocols. The PCR reactions were subjected to the following cycle: denaturation (94°C, 30 sec), annealing (61°C for USP22 and  $\beta$ -actin, 30 sec) and extension (72°C, 30 sec); USP22: 37 cycles,  $\beta$ -actin: 30 cycles.

### Western blot

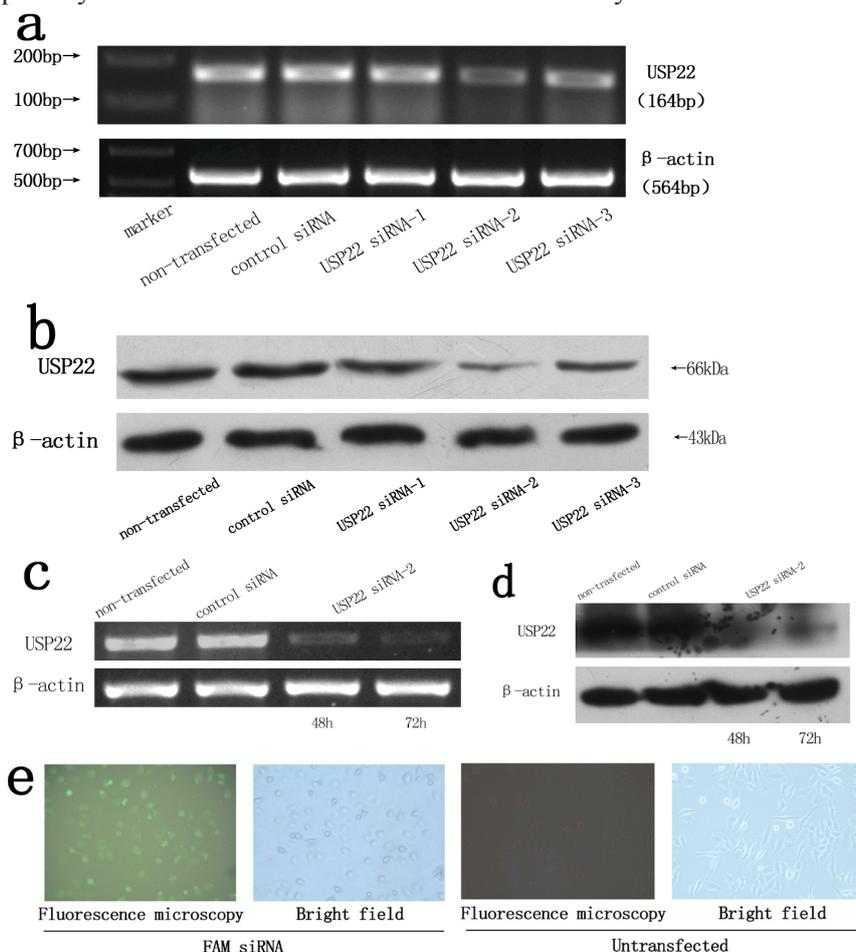
Cells were harvested in ice-cold phosphate-buffered saline (PBS) containing 100 mM sodium orthovanadate and were solubilized in lysis buffer (50 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 100 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1% Nonidet P-40, 5 mM cocktail; pH 7.4). The admixture was centrifuged at 12,000  $\times$  g for 20 min, and the supernatant was collected. The protein concentration was determined using the BCA assay kit (Pierce, USA), and each sample contained 100  $\mu$ g/20  $\mu$ l protein. The sample was mixed with sample buffer containing  $\beta$ -mercaptoethanol and bromophenol blue. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transblotted to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, USA). The blots were incubated at 4°C overnight with the first antibody and subsequently at 37°C for 1 hour with the second antibody. The bands were visualized by chemiluminescence. An image-analysis system was used to semi-quantify the intensities of the bands.

### Transfection of siRNA

HepG2 cells were seeded in a 6-well plate at a concentration of  $4 \times 10^5$  cells per 2 ml in the medium of RPMI-1640 supplemented with 10% FBS and 100  $\mu$ g/ml each of penicillin and streptomycin. After 24 hours, the medium was replaced with fresh medium without antibiotics or FBS. Meanwhile, siRNA or a negative control oligonucleotide was transfected into the cells with Lipofectamine 2000 according to the manufacturer's instructions. After incubation for 4-6 hours, the medium containing the siRNA-Lipofectamine 2000 complexes was replaced with fresh RPMI-1640 containing 10% FBS. Then, the cells were cultured for subsequent experiments. All siRNAs were obtained from Shanghai GenePharma Co., Ltd., and the three specific sequences for silencing were: human USP22 siRNA-1, sense 5'-GCCAGGACUACAUCUAUGATT-3', and anti-sense 5'-UCAUAGAUGUAGUCCUGGCTT-3'; siRNA-2, sense 5'-CACGGACAGUCUCAACAAUTT-3', and anti-sense 5'-AUUGUUGAGACUGUCCGUGTT-3'; siRNA-3, sense 5'-CUGCAAAGGUGAUGACAAUTT-3', and anti-sense 5'-AUUGUCAUCACCUUUGCAGTT-3'. The transfection efficiency was examined by transfection of negative control FAM siRNA, and the subsequent monitoring of the cells was performed using fluorescence microscopy. The effect of RNA interference was analyzed by RT-PCR and Western blot analyses.

### Cell viability assay

Cell viability was determined using the methyl thiazolyl



**Figure 1.** The USP22 siRNA-2 was optimal in silencing the expression of USP22. (a,b) siRNA-1, -2 and -3 were transfected into HepG2 cells (20 nM) for 24h; subsequently, the USP22 mRNA levels were quantified by RT-PCR and the protein levels were quantified by Western blot. These results demonstrate that siRNA-2 most efficiently inhibited the expression of USP22. (c,d) The effect of interference could last at least 72 h, as determined by RT-PCR and Western blot analyses. (e) After transfection with FAM siRNA, the cells were detected by fluorescence microscopy, verifying a high efficiency of transfection. Untransfected cells were examined as control.

terazolium (MTT) assay. Cells were seeded on a 96-well plate ( $2.5 \times 10^3$  cells/well) one day prior to siRNA transfection. The following day, the cells were transfected with USP22 siRNA or control siRNA. MTT (20  $\mu$ l of 0.5 mg/ml) was added to cells at different time points after the transfection. Then, the cells were incubated at 37°C for 4 h to allow the yellow dye to be transformed into blue crystals. The medium was removed, and 200  $\mu$ l of dimethyl sulfoxide (DMSO) was added to each well to dissolve the dark blue crystals. Finally, the optical density was measured with a microtiter plate reader at 570 nm. Six replicates were prepared for each condition. The mean values were calculated, and a growth curve was drawn.

#### Cell cycle analysis of by flow cytometry

Different groups of HepG2 cells (non-transfected group, control group and USP22 siRNA group) were prepared 48 hours after siRNA transfection. A total of  $1 \times 10^6$  cells was pelleted by centrifugation and washed twice with PBS. Then, the cell pellets were resuspended in 500  $\mu$ l of ice-cold 70% ethanol and incubated at 4°C overnight. The fixed cells were centrifuged, and the pellets were washed with PBS. After incubation with 100  $\mu$ l RNase A (10 mg/l) for 30 min at 37°C in the dark, the cells were resuspended in 400  $\mu$ l PI (50 mg/l) and placed at 4°C in the dark for 30 min. The stained cells were analyzed using a FACSCalibur flow cytometer (Becton-Dickinson, USA).

#### Annexin V-FITC/PI double-labeled detection of apoptosis

The protocol was based on the use of fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) staining according to the manufacturer's instructions (Annexin V-FITC kit; Bio-Rad). Analysis by flow cytometry was performed to differentiate among the types of cell death. Cells that were Annexin V-positive and PI-negative were classified as apoptotic or early-stage apoptotic cells. Briefly, cells were digested with 0.25% trypsin, washed three or four times and then stained for 15-20 min until the cells were completely digested. After the cells were digested, they were washed with PBS. Unfixed cells were stained by incubating them in the Annexin V-FITC reaction reagent (10  $\mu$ l Annexin V-FITC, 5  $\mu$ l propidium iodide) at 37°C for 30 min in the dark. The stained cells were analyzed by flow cytometry using a FACSCalibur Flow Cytometer (Becton-Dickinson, USA).

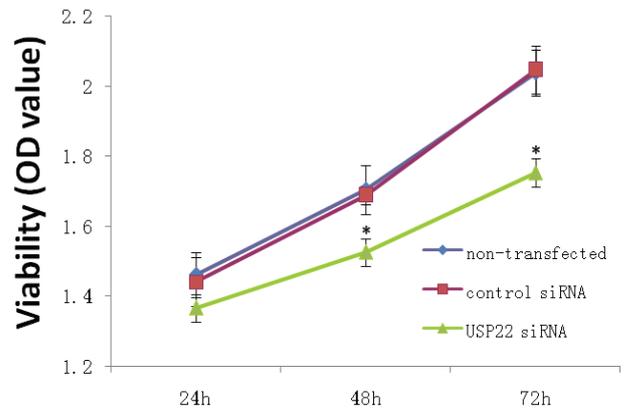
## RESULTS

#### Suppression of USP22 Expression in HepG2 cells by siRNA transfection

Three siRNAs (siRNA-1, -2 and -3) were used to silence the expression of USP22 in HepG2 hepatoma cells. The siRNAs and a FAM-negative control oligonucleotide (20 nM) were transfected into HepG2 cells for 24 h. The transfection efficiency was determined by fluorescence microscopy (Fig. 1e), and effect of the transfection was determined by RT-PCR and Western blot analysis (Fig. 1a, b, c and d). The RT-PCR results revealed the presence of 164-bp bands in each group and the Western blot results displayed 64kDa bands in each group. The results of our study showed that the USP22 siRNA-2 was most efficient in silencing the expression of USP22.

#### Down-regulation of USP22 by siRNA reduces viability of HepG2 cells

To explore the effects of down-regulating USP22 by siRNA on the viability of HepG2 cells, we monitored HepG2 cell growth by the MTT assay at different time points (24 h, 48 h, 72 h) after transfection of the cells with either USP22 siRNA or control siRNA (Fig. 2). Our data demonstrated that transfection with USP22 siRNA markedly reduced cell growth compared to transfection with the control siRNA.



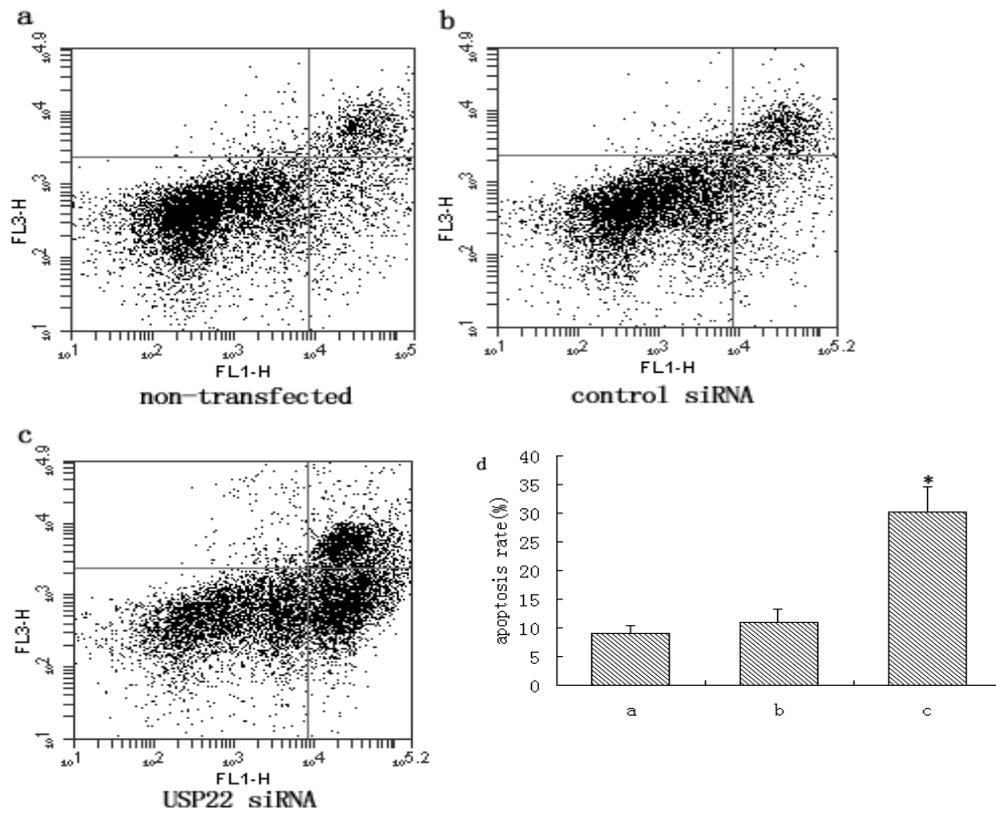
**Figure 2.** USP22 siRNA transfection inhibits HepG2 cell proliferation. HepG2 cells were transfected with USP22 siRNA for 24, 48, and 72 hours, and then the MTT assay was performed to determine cell viability. The cell viability in the USP siRNA group was reduced compared to the non-transfected and control siRNA transfected groups (\* $p < 0.05$ ).

#### Promotion of cell apoptosis and inhibition of the cell cycle in HepG2 cells after USP22 siRNA transfection

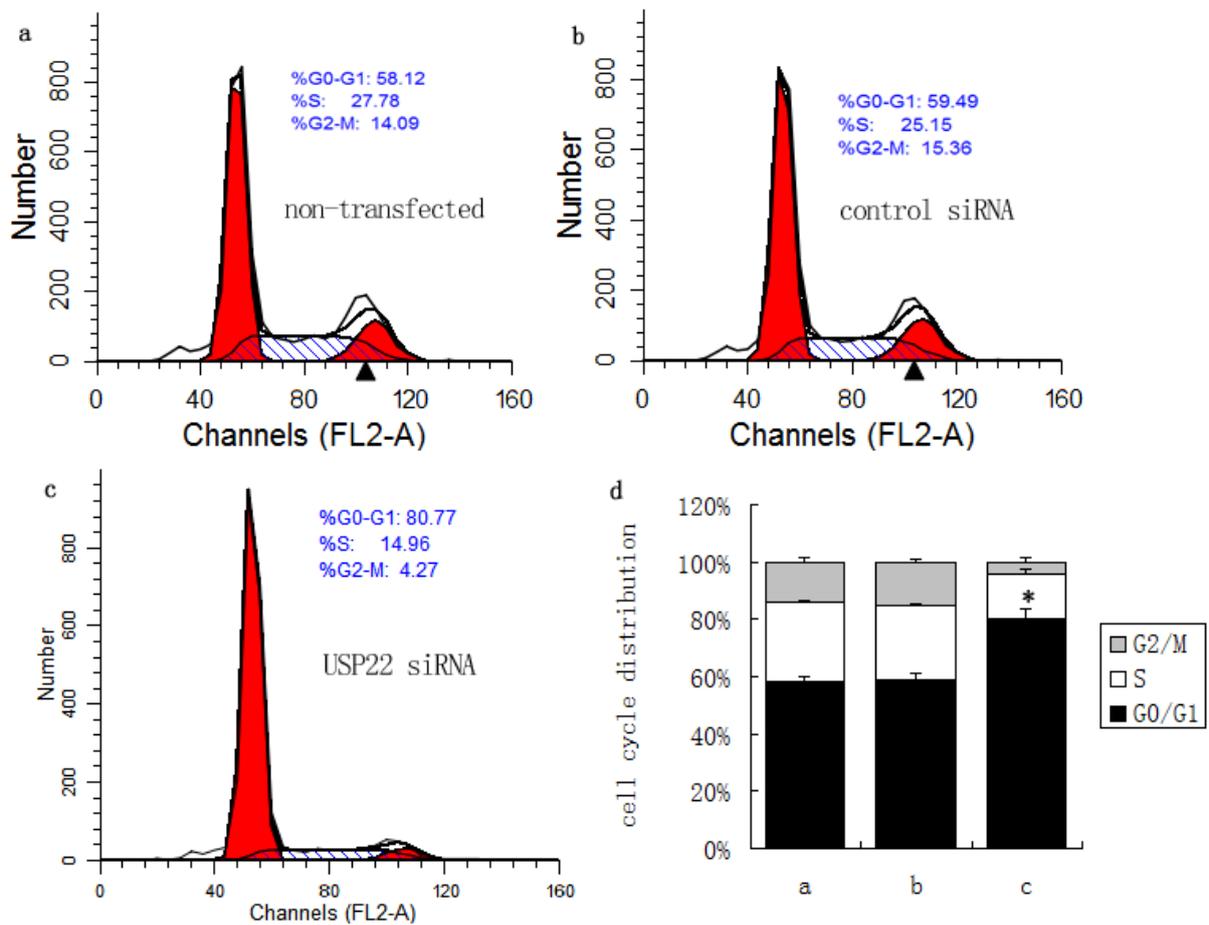
We examined the number of apoptotic cells and cell cycle distribution after USP22 siRNA transfection by flow cytometric analysis. A significant increase in the number of apoptotic cells and cells undergoing G0/G1 cell cycle arrest was observed in siRNA-USP22 transfected cells compared with the non-transfected cells and negative siRNA-transfected cells (Fig. 3 and Fig. 4). After transfection with siRNA-USP22, the rate of apoptosis increased from 9.23% and 11.62% to 30.32% (Fig. 3). The number of cells in the G0/G1 phase was 80.77% in the siRNA-USP22 transfected cells; this was a marked increase from the 58.12% in non-transfected cells and 59.49% in negative siRNA-transfected cells (Fig. 4). These results indicate that USP22 may facilitate tumor cell growth by regulating apoptosis and cell cycle progression in HepG2 cells.

#### Increased expression of the p15 and p21 genes and decreased expression of the cyclin D2 gene were detected after USP22-siRNA transfection in HepG2 cells

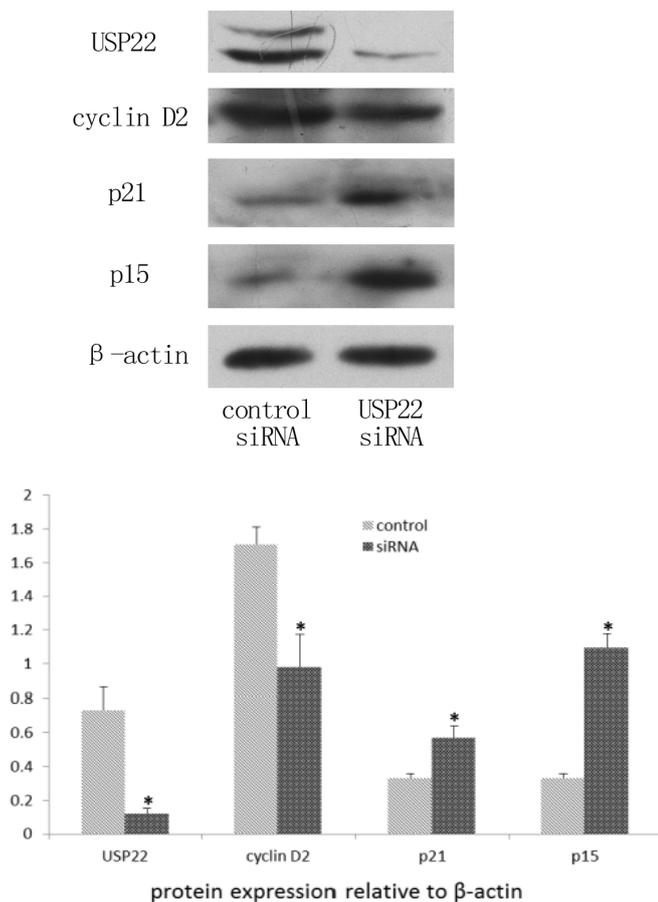
p15, p21 and cyclin D2 are responsible for cell cycle progression. Thus, we examined the variation in the expression of p15, p21 and cyclin D2 in siRNA-USP22-transfected cells and control-siRNA-transfected cells. The Western blot results demonstrated that p15 and p21 were up-regulated and cyclin D2 was down-regulated after USP22 knock-down in HepG2 cells (Fig. 5). This result suggests that USP22 may affect cell cycle progression by down-regulating p15 and p21 expression and up-regulating cyclin D2 expression.



**Figure 3.** Knock-down of USP22 facilitates apoptosis in HepG2 cells. (a-c) After transfection with siRNA for 48 h, the distribution of apoptotic cells was measured by flow cytometric analysis, and the apoptosis rates in different groups are shown in the bar graph. (d) The apoptosis rate in the USP22-siRNA-transfected group was higher than the rates in the non-transfected and control siRNA groups (\* $p < 0.05$ ).



**Figure 4.** Knock-down of USP22 induces G0/G1 cell cycle arrest in HepG2 cells. (a-c) After transfection with siRNA for 48 h, the cell cycle distribution of each group was determined by flow cytometric analysis. (d) The USP22-siRNA group has a significant increase in the number of cells in the G0/G1 phase compared to the non-transfected and control siRNA groups (\* $p < 0.05$ ).



**Figure 5.** Effect of USP22 on the expression of cell cycle-related regulators in HepG2 cells. Expression of cyclin D2, p21 and p15 was monitored by Western blot analysis 48 h after transfection. Differential expression of each gene was presented (\* $p < 0.05$ ).

## DISCUSSION

USP22 is a recently identified novel human deubiquitinating enzyme. Elevated expression of USP22 can predict shorter intervals of tumor recurrence, distant metastasis, therapeutic failure and poor prognosis in patients with many types of cancer (3,7,13,16). Zhang *et al.* (15) silenced USP22 by RNAi in the human non-small cell lung carcinoma line H1299 and subsequently observed that Myc-mediated transformation was inhibited and the cell cycle was arrested in the G1 phase. Similar results in recent studies have led to the suggestion that silencing USP22 could suppress proliferation of tumor cells (6,8,12). Because little is known about the impact of USP22 knock-down in human hepatoma cell lines, we investigated whether USP22 plays a similar role in the growth of the human HepG2 hepatic cancer cell line.

In this study, expression of USP22 was observed in the HepG2 cell line. Subsequently, USP22 expression was effectively depleted in HepG2 cells by siRNA, as detected by RT-PCR and Western blot analyses. USP22 depletion resulted in cell cycle arrest at the G0/G1 phase, with a concomitant decrease in the S and G2/M phases in HepG2 cells.

We also observed that the cell cycle inhibitors p15 and p21 were increasingly expressed upon USP22 knock-down and that cyclin D2, identified as a positive cell cycle regulator, was down-regulated upon USP22 inactivation. USP22 is known to down-regulate c-Myc and its targeting genes, such as JPO1, cyclinD2, ODC, CAD and MTA1 (15). It

can be speculated that USP22 regulates the cell cycle via the c-Myc/cyclin D2 pathway in HepG2 cells. Moreover, p21 and p15 may be the other effectors responsible for the cell cycle arrest induced upon knock-down of USP22. p21, which is identified as a target gene of p53, inhibits the kinase activity of the cyclin-CDK complex and inhibits cellular proliferation in response to DNA damage. It has been suggested that USP22 is required for the transcription of the p53 target genes p21, PIG3 and PUMA, which were down-regulated upon USP22 depletion in H1299 cells (15). p21 may not be an effector of USP22 depletion-induced cell cycle arrest because it requires USP22 function. However, inverse results were shown in another study, in which knock-down of USP22 up-regulated the expression of p53 and p21 in human bladder cancer EJ cells(8). p21 was identified as an independent survival prognostic factor for patients with hepatocellular carcinoma (HCC) after resectioning(5). An inverse correlation between p21 expression and HCC tumorigenesis has been described in several reports. Boyko *et al.* (5) described a potential regulated pathway in which USP22 regulates p21 expression by altering Far Upstream Element (FUSE)-Binding Protein 1 (FBP1) ubiquitination. USP22 depletion leads to increased FBP1 ubiquitination and decreased FBP1 protein occupancy at the p21 gene, causing increased p21 expression in normal human fibroblasts and HeLa cells. FBP1 is required for tumor growth in hepatocellular carcinoma, and upon FBP1 knock-down, p21 and p15 were up-regulated and cell cycle arrest was observed (10). p15 could inhibit the activity of cyclin-dependent kinase 4/6(CDK4/6) and induce cell cycle arrest(14). Down-regulation of p15 expression in HCC tumor samples was also described as a result of 5' CpG island methylation in the p15 promoter (11). Thus, our results implied that USP22 is responsible for cell-cycle progression in HepG2 cells by facilitating the c-Myc/cyclinD2 pathway and inhibiting p21 and p15 expression; further investigation is required to elucidate the exact mechanisms.

Furthermore, based on the results of the MTT assay and FACS analyses, we observed that knock-down of USP22 induces cell growth inhibition and facilitates apoptosis.

In summary, the siRNA-mediated knock-down of USP22 could effectively induce cell cycle arrest by regulating target molecules, such as cyclinD2, p21 and p15, and could also inhibit HepG2 cell growth. Consequently, USP22 may be an attractive therapeutic target for the treatment of liver tumors. Further investigations should explore the exact role of USP22 in tumor progression, as well as the specific regulators and effectors of USP22.

## ACKNOWLEDGMENT

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